Alginate Strings and Their Applications in Spinal Cord Regeneration

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DEDICATION

I would like to dedicate this work to my mother who believed in my abilities and pushed me to achieve perfection in whatever I did.

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ABSTRACT

Alginate Strings and Their Applications in Spinal Cord Regeneration

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Spinal cord injury leads to severing of neurons of the central nervous system (CNS) that are responsible for communication between the brain and other parts of the body. The presence of inhibiting factors following injury limits neuronal regeneration leading to paraplegia or quadriplegia depending on the level of the injury. The goal of this study was to design and develop a graft that would aid in neuronal adhesion as well as enhance neuronal regeneration and also act as a physical bridge at the site of injury. Alginate a polysaccharide derived from brown sea weed was employed in making the graft because of its ability to form gels in the presence of a polycation solution and also allows modification with growth permissive peptides. YIGSR a pentapeptide of laminin has been found to enhance neuronal adhesion. The YIGSR modified alginate graft was made in the form of strings so that it could act as a bridge at the site of injury. Rat abdominal fibroblasts were genetically engineered by *ex vivo* gene therapy to secrete human brain derived nerve growth factor (BDNF) that would enhance neuronal growth. These cells were incorporated in the modified alginate solution before they were made into strings, and the strings were then coated with a high molecular weight polyamine. This polyamine coating would prevent high molecular weight immunoglobulins like IgG from entering the graft interacting with the fibroblasts while allowing the lower molecular weight BDNF to escape to the outside. This graft could then be frozen and stored under liquid nitrogen and thawed when needed, and placed at the injury site without immune suppression.

1. INTRODUCTION

1.1 Treatment of Spinal Cord Injury

Spinal cord injury (SCI) affects nearly 200,000 people in the United States and approximately 11,000 people sustain new spinal injuries every year[1]. Most SCIs are incurred as a result of auto accidents, sports injuries, falls or industrial mishaps[1]. Nearly 60% of those affected are 30 years or younger and are mostly men. SCI has a tremendous impact on a person, physically, emotionally and socially. Following spinal cord injury there is disruption of communication between the brain and other parts of the body, and messages are no longer transmitted across the injured area[2]. SCI can occur at any level and the bodily functions that may be lost or altered depend on the level of injury. The higher the injury, the more movement and sensation will be lost or altered. An injury at the cervical (neck) level may cause paralysis of both arms and legs resulting in quadriplegia, while lower injuries like those at the thoracic (upper-back) level may affect only the lower part of the body causing paraplegia. Injuries are also classified as complete or incomplete depending on whether there is total or partial loss of sensation and movement below the level of injury. SCI can also result in loss of bowel and bladder control. This type of debilitating injury requires lifelong care and rehabilitation at a cost of about 1

million dollars in the case of quadriplegics[1].

The reason central nervous system (CNS) neurons do not regenerate following an injury may be due to the poor intrinsic regenerative ability of the neurons themselves, presence of a glial scar and circulating macrophages and proteoglycans[3]. Various strategies have been tried to aid in neuronal regeneration following injury, some of them being peripheral nerve transplantation, stem cell transplantation, provision of guidance channels, release of neurotrophic factors via *ex vivo* gene therapy, etc. While several of these strategies have shown positive results the general consensus would be for developing a treatment method that would combine many of the above mentioned strategies to obtained the desired results.

1.2 *Ex Vivo* Gene Therapy in Spinal Cord Injury Treatment

Neurotrophic factors are known to prevent neuronal death as well as enhance axonal regeneration following injury[4]. Direct application of neurotrophic factors at the site of injury is not very effective since multiple doses would be required leading to repeated invasive procedures. Systemic administration would also be ineffective since the neurotrophic factors would not cross the blood brain barrier and ultimately not reach the target site. Ex vivo gene therapy would help to circumnavigate these problems and deliver the factors at the injury site in an

effective manner. *Ex vivo* gene therapy involves transplanting cells that have been genetically engineered to release neurotrophic factors, at the site of injury. In an ideal situation the patients own cells would be used for the *ex vivo* gene therapy to prevent graft rejection by the host immune system, but this process would not only be expensive but also time consuming. Since treatment needs to be available shortly following injury to enhance nerve regeneration non-autologous grafts have been developed. Cell lines have been developed that were modified to express different neurotrophic factors, and stored so they would be readily available[5, 6]. Transplantation of non-autologous cells would require the patient to be under immune suppressive therapy in order to avoid graft rejection by the immune system, which in turn would lead to complications such as infections. This would lead to the graft being less or non effective. These modified cells could be protected from the host immune system by encapsulating them in a polymer matrix such as collagen[7], Matrigel[8] and alginate[9].

1.3 Alginate

Alginate is a polysaccharide obtained from brown sea weed[10]. It contains β-(1-4)-linked D-mannuronic acid (**M**) and α-(1-4)-linked L-guluronic acid (**G**) residues. Alginate is therefore a polymer that is linear and unbranched. The D- mannuronate

chain forms a 3-fold-left handed helix with weak intramolecular hydrogen bonding between the hydroxyl group in position 3 and the following oxygen ring[11]. Alginate forms gels with divalent cations like calcium, barium, etc and certain trivalent cations like iron and aluminium, via inter-chain cross linking of the carboxylate groups on the guluronate units. The properties of the gel are governed by the sequence and composition of the monomer chains. The greater the content of G monomers the stronger is the gel formed. Alginate hydrogels can be prepared by adding a solution of sodium alginate to a cation solution which causes the crosslinking. Alginate belongs to a group of compounds that have been generally regarded as safe by the FDA. It has also been used in tissue engineering for cell encapsulation and various other functions[9, 12]. Alginate hydrogels can also be coated with poly cations such as poly-L-ornithine which acts as a semipermeable membrane regulating the substances that go in and out of the hydrogel. Thus this coating can prevent the immunoglobulins of the host from affecting the encapsulated cells and also allow the diffusion of the neurotrophic factors released from the cells. Thus immune suppression of the host and its complications can be avoided. Alginate can also be modified with specific peptides that would further make the surface of the hydrogel favourable for cell adhesion and regeneration[9].

1.4 Statement of the Problem

The aim of this thesis is to develop a graft that could be implanted in the spinal cord following spinal cord injury (SCI) that would aid neuronal regeneration. The goal is to provide a surface that supports neuronal cell adhesion and promotes neuronal regeneration. The alginate graft should act as a bridge at the site of the injury and provide an environment that is favorable for cell adhesion and growth. The graft should be stable and in a size range that is compatible for grafting in the spinal cord. The graft should be non-toxic and safe for human transplant. The graft should be stable in the body over a period of time for it to act as bridge in neuronal regeneration. The method used for developing this graft should be repeatable. The specific goals of this thesis were:

1. To develop a method for fabricating alginate hydrogel strings. The aim is to develop alginate strings that would acts as a bridge across the site of spinal injury and would be stable.
2. To modify the surface of the alginate strings with growth permissive peptides. The aim was to develop a graft whose surface would promote neuronal cell adhesion and regeneration. Use *in vitro* techniques to determine the effect of peptide modification of alginate on neuronal cell adhesion and growth.

3. To determine the effect of peptide type and concentration on neuronal cell adhesion. The aim was to modify alginate with different concentrations of specific peptides and determine *in vitro* the optimum concentration of peptide modification that ensures neuronal cell adhesion.
4. To develop a method to encapsulate genetically engineered cells in the strings. The aim is to encapsulate genetically engineered cells that secrete neurotrophic factors in the strings. The object was to determine the effect of encapsulation on cell survival.
5. To test *in vitro* transgene expression using specific staining kits. The aim is test transgene expression by the cells encapsulated in the alginate strings and also study *in vitro*, the effect of the neurotrophic factors on nerve cell growth and differentiation.
6. To develop a method to store the alginate strings for immediate use. The aim was to freeze and reconstitute the alginate strings with the modified cells. This can be tested *in vitro* by freezing and thawing the strings containing the modified cells and checking for cell survival and transgene expression.

2. BACKGROUND AND LITERATURE REVIEW

2.1 Spinal Cord Injury

Spinal cord injury (SCI) affects nearly 200,000 people in the United States and approximately 11,000 people sustain new spinal injuries every year. Most SCI's are incurred as a result of auto accidents, sports injuries, falls or industrial mishaps[1]. Mostly occurs in young adults particularly males as a part of sports injury, automobile accidents, etc. SCI has a tremendous impact on a person, physically, emotionally and socially. Following spinal cord injury there is disruption of communication between the brain and other parts of the body, and messages are no longer transmitted across the injured area[2]. The extent of disruption in communication depends upon the severity and the level of the injury. The human spinal cord is a bundle of nerves about a foot and a half long, and extends from the brain to the lower back. The spinal cord acts in relaying messages from the brain to the other parts of the body and also receives information from all over the body which is in turn transmitted back the brain. Since nerve fibers are very sensitive they are protected by the backbone which consists of 33 vertebrae which are classified into 5 regions[13].

Following trauma there is axonal injury leading to death of disconnected

neurites and vascular damage leading to hypoxia. The inflammatory response following injury leads to the appearance of macrophages at the site which in turn contribute to the scar formation[14]. The scar formation by the macrophages, astrocytes and microglia is a result of the body re-establishing the blood brain barrier thereby preventing further injury to the CNS[15]. The cells and connective tissue that make up the scar have been found to release both growth promoting and inhibiting molecules. Regeneration of central nervous system neurons basically depends on the severity of the injury to the neurons. If the somata of the neurons are involved in the lesion then the neurons die immediately. If the injury is only an axotomy, transection or severing of an axon, without injury to the soma the cells would die by apoptosis days or weeks after the injury[16]. Once the distal nerve segments are separated from their cell bodies they start to degrade and are taken up by macrophages over a period of time (Wallerian degeneration)[17]. Degeneration of the axon causes loss of contact with the surrounding astrocytes and oligodendrocytes leading to loss of functions that are dependent on the activity of the neurons. To combat this, the neurons try to grow into the lesion but this process is abrupt and there are no lengthy neurites that bridge the lesion. This is due to the retraction of the growth cones when they come in contact with the injury and also

due to myelin removal.

Earlier it was believed that CNS neurons lacked the ability to regenerate. Recent research has shown that the CNS neurons do have the ability to regenerate, but the ability of the neurons to regenerate, bridge the lesion and regain their functionality depends on various factors. The rate of regeneration not only varies between different CNS neurons but also within different branches of the same neuron. Supply of neurotrophic factors plays a vital role in neuronal regeneration. Following CNS neuronal injury there is the presence of neurotrophic factors, which are essential for the survival of the axotomized neurons. The percentage of surviving axotomized neurons is reduced by about 30% within the first 7 days, or even to 50% if there is circulating antibodies to brain derived nerve growth factor (BDNF) [18]. Experiments performed by Giehl *et al*[18] have demonstrated that infusion of BDNF directly into the cortex can help to save these axotomized neurons showing that there is a lack of endogenous trophic factors for the axotomized corticospinal neurons. Therefore BDNF along with other neurotrophic factors plays a vital role in neuronal regeneration.

Axonal regeneration is also dependant on regeneration associated genes (RAGs). Total axonal growth following regeneration depends on the level as well as

the types of RAGs expressed and also on the axonal environment. Following injury to CNS neurons expression of RAGs remains unchanged in most of the dorsal root ganglia (DRG). One of the RAGs, Growth associated protein-43 (GAP-43), is important in axonal sprouting[19]. DRG neurons that express high levels of GAP-43 tend to grow several mm/day provided they have a growth permissive environment, but most DRG neurons following injury express very low levels of GAP-43 leading to a slow rate of axonal regeneration.

Another factor that regulates axonal regeneration is the extracellular matrix (ECM). ECM molecules like laminin, fibronectin, proteoglycans, collagen, etc. play an important role in neurite outgrowth. In the CNS these ECM molecules are synthesized by the astrocytes. Laminin is a promoter of neurite outgrowth whereas proteoglycans inhibit them. Laminin is a 900kDa complex extracellular protein; composed of three different polypeptide chains termed α, β and γ. These chains are linked together in various combinations to form 15 known isoforms or types of laminin. Each isoform has distinct characteristics and functions. Isoforms are known to change in normal tissues at various stages of development but they also have been found to shift in the presence of several invasive cancers. Quantitatively it is one of the most abundant glycoprotiens present in the basement membrane. It helps in

modulating several key biological activities including cell adhesion, migration, cell survival and gene expression[20, 21]. Variability in the spatial and temporal expression of laminins suggests that different laminins perform distinct functions. The expression levels of the extracellular matrix (ECM) glycoprotein laminin-1 are high along axonal tracts in the developing cerebellum, suggesting that laminin-1 plays a major role in cerebellar granule cell pathfinding. Laminin-1 has been found to be one of the key molecules in the CNS following injury[22].

2.2 Strategies in Treating Spinal Cord Injury

Various strategies have been used to treat spinal cord injuries and several different approaches are also currently being tested *in vitro* and in animal spinal injury models. Some of the strategies that are currently being explored include grafting of peripheral nerves to the lesion, providing growth factors, stem cell transplants, supplying antibodies to growth inhibiting molecules, guidance channels like collagen tubes, *ex vivo* gene therapy with encapsulated cells and combination therapies. Some of these strategies are discussed below.

2.2.1 Cell Grafting

The axons in the peripheral nervous system, just like the CNS, regenerate only if their cell bodies are intact. Shortly after axonal injury there is cell body

swelling which is followed by Wallerian degeneration where the distal portion of the axons and myelin sheath start to degrade. Finally the intact neurolemma cells divide and regeneration tubes are formed through which new axons and eventually myelin will reform. Axotomized neurons start to express growth associated proteins like GAP-43, cytokines and other neuropeptides. Schwann cells, whose primary function is myelination of axons, start expressing cell adhesion molecules and extracellular matrix proteins that would aid in axonal regeneration[23]. Since Schwann cells act as guidance channels for regenerating axons in the PNS they were transplanted in the CNS to verify if they had the same effect. Trials with injections of cultured Schwann cells or grafts containing Schwann cells in injured spinal cord have shown increase in axonal sprouting[24]. If Schwann cells are transplanted immediately following spinal injury they survive better and transected axons are seen growing into the guidance channels[25]. Though there was axonal sprouting only a small percentage (1%) were found to enter the host tissue.

Transplants of embryonic rat brain and spinal cord tissue have been shown to survive and differentiate in damaged adult rat spinal cords[26]. Transplantation of an intact brain stem provides a source of neurotransmitters like serotonin or nor-adrenaline for the denervated spinal cord and they can also act as a bridge for the

regenerating axons to grow along[27]. The fetal grafts also provide neurons onto which the host axons can synapse, a process that would help relay neural signals and the graft may also provide glial cells that would aid in remyelinating the damaged axons. Transplantation of fetal neural tissue in the adult spinal cord has been less successful than in the neonatal spinal cord. It has been found that though the host neurons synapse with the graft, they fail to project through the lesion and the donor neurons also extend from the graft for only a few millimeters[28]. Injured adult neurons may require neurotrophic factors in addition to the graft to aid in regeneration.

Olfactory neurons are the only kind of neurons that are produced and continue to divide throughout adult life[29]. The olfactory ensheathing cells (OECs) are specialized glial cells that encourage the growth of axons from the mucosa and they possess the characteristics of both Schwann cells as well as astrocytes. Unlike the Schwann cells that are confined to the PNS where these OECs accompany the olfactory axons to the CNS and provide guidance for the axons to their correct positions in the olfactory glomerulus. These are the only glial cells that have the capacity to cross the PNS-CNS boundary and can also myelinate axons *in vitro*[30]. It was found that grafting of the olfactory ensheathing cells resulted in axons growing

through the lesion, and continued regeneration through the denervated region. The olfactory cells also caused the myelination of both individual as well as groups of axons forming fascicles[31]. The advantage of this type of treatment would be that cells from the patient's own olfactory mucosa could be cultured, thereby circumventing the problems of graft rejection and immune suppression. The limiting factor of this kind of therapy would be that the graft would not be readily available following injury.

Another kind of cell line that has been under study for grafting to combat spinal cord injury is the embryonic stem cells. The reason that stem cells are attractive for grafting is that they are totipotent cells and can be made to differentiate into any phenotype with the addition of specific differentiating factors. These cells can also be maintained in culture and used as a source of neural cells[32]. In 1993 Pleasure et al implanted neurons, prepared from NT2N cell line by treating them with retinoic acid, into the spinal cord of mice[32]. The neurons not only survived in the host for a prolonged period of time but also managed to mature and show neurite outgrowth. This approach could be promising in treating spinal injury since these cells are readily available for grafting[33].

2.2.2 Antibodies to Growth Inhibiting Molecules

Following injury oligodendrocytes and astrocytes are present as a result of the inflammatory response. Both these cell types have growth inhibitory molecules for axons and axons can only regenerate if they either have no receptors to these inhibitory molecules or if they can bypass the signals released from them. Embryonic neurons are able to grow over oligodendrocytes while mature sensory axons show an increase in the calcium level in their growth cones leading to their collapse. Astrocytes produce proteoglycans that inhibit neuronal regeneration by blocking the growth promoting characteristics of laminin and other ECM molecules[34]. Inhibiting factors released by oligodendrocytes and myelin can be combated using antibody IN-1, which has proven effective *in vitro*[35] and also when used in adult rat spinal injury models[36].

Astrocytes produce proteoglycans that are inhibitory to the growth promoting effects of laminin and other basement membrane molecules. The enzyme that has been shown to be efficient against the proteoglycan mediated inhibition is bacterial Chondroitinase ABC. This enzyme acts by cleaving the glycosaminoglycan (GAG) side chains on the chondroitin sulfate proteoglycans (CSPGs)[37]. This enzyme however does not fully digest the GAG chains leaving behind side chains which,

even though they are less inhibitory than entire GAG chains, reduce the axonal regeneration capacity[38]. Currently research is being done on an improved target specific enzyme Xylosyltransferase-1 that acts on the m-RNA of the enzyme that initiates the GAG formation on the CSPGs[39]. Combining neurotrophic factors with these enzymes would help to enhance axonal regeneration.

2.2.3 Neurotrophic Factors

Neurotrophic factors are growth factors produced mainly in the embryonic stage in humans, to aid in the growth and development of the CNS. These factors are not generally produced during adult life unless there is an injury to the CNS. Studies were performed on the nerve growth factor (NGF) by Scott D Jr et al as early as 1963[40]. Further studies have shown that there are growth factors other than NGF like neurotrophin-3 (NT-3), brain derived nerve growth factor (BDNF), glial cell derived nerve growth factor (GDNF) and ciliary neurotrophic factor (CNTF). Studies using these nerve growth factors to treat adult rat spinal cord injury, either singly or in combination, have shown that they not only help to prevent atrophy of neurons but also help in their regeneration[41, 42, and 43]. Since these factors do not cross the blood brain barrier they have to be administered locally rather than systemically. The problem with local administration of these factors would be that

repeated invasive techniques have to be employed and this problem can be overcome by the local transplantation of cells that are genetically engineered to release specific nerve growth factors.

2.2.4 Nerve Guidance Channels

The aforementioned studies show that the CNS neurons have the capacity to regenerate if the environment can be made growth permissive. Even though these studies have proved that axons can sprout into the lesion they are not conclusive if the sprouting axons would bridge the lesion and synapse with the neurons across the gap. For this purpose guidance channels in the form of tubes that have been used in peripheral nerve repair[44] are also being employed in spinal cord injuries[45, 46, and 47]. The use of tubes in the repair procedure allows the regulatory factors and the cells from the distal stump to remain in direct contact with the site where the regeneration occurs. It also provides a direction for the axons crossing the gap and also helps to isolate the injury site from exogenous cells and tissues[48]. The last fact could be beneficial in that it would prevent the regenerating neurons from being exposed to macrophages and various other inhibitory factors, at the same time it could also prove to be detrimental by not allowing the growth factors that are released to reach the growing neurons. Among the different types of tubes that have

been employed for treating axonal regeneration in the CNS collagen tubes seem to be the best suited[48]. The advantages of using collagen are that it is non-toxic, non antigenic, flexible, biodegradable, permeable and also promotes regeneration[49, 50]. Studies have also show that the use of collagen tubes helps to reduce the glial response that occurs after injury[51].

2.2.5 Ex Vivo Gene Therapy

Another approach to treating spinal cord injury is to transplant cells that have been genetically engineered to release neurotrophic factors at the site of the lesion. As mentioned earlier systemic administration of neurotrophic factors is ineffective in treating spinal cord injury since these factors cannot cross the blood brain barrier. Implantation of Schwann cells that were genetically engineered to express BDNF in adult rat spinal injury models led to increase in neuronal regeneration across the complete transection[8]. Fibroblasts are the most commonly used cell lines for such modifications since they can be harvested easily and are also a robust cell line that is easy to culture. Transplantation of primary rat fibroblasts, which were modified to express NGF, into adult rats with spinal injury led to survival and regeneration of sensory, motor and nor-adrenergic neurites[5]. Transplantation of fibroblasts that express neurotrophin-3 (NT-3) in adult rats with dorsal cord lesion led to partial

functional recovery[52]. These genetically engineered cells have also been found to promote neuronal regeneration when transplanted months after injury which proves that they induce existing neurons to extend neurites into the lesion. This approach could be employed to modify cells to express other neurotrophic factors like BDNF, ciliary neurotrophic factor (CNTF) and glial cell line derived neurotrophic factor (GDNF). Primary rat abdominal skin fibroblasts that were genetically modified to release BDNF were transplanted into adult Sprague Dawley rats with partial cervical hemisection[2]. The result was rubrospinal tract regeneration that was observed for a period of up to 2 months after lesion and transplantation. The study also showed that several neurons showed sprouting into the graft and beyond, into the lesioned area and spinal segments. Behavioral studies performed on these animals also showed that they had significant recovery of forelimb function in the presence of the BDNF secreting fibroblast graft[9]. Similar studies have been conducted using neuronal stem cells that have been genetically modified to express NT-3 in spinal cord injury repair[53]. This test showed that the neuronal stem cells not only survived but also produced NT-3 in adult rat spinal cords. These stem cells also differentiated into neuronal and glial cells and migrated in the cord for long distances. For application in humans the hosts own cells could be genetically modified to express the desired

neurotrophic factor. This however would not be feasible if the graft had to be applied immediately following injury. In such situations ready made non-autologous (allografts) grafts have to be employed. Grafting cells that are foreign to the host however might lead to strong host immune reaction warranting the need for a rigid immune suppressive protocol. Such an immune suppressive therapy not only makes the host susceptible to various infections but could also reduce the effectiveness of the graft. The way to circumvent this problem would be to encapsulate these genetically modified cells in a selectively permeable matrix, which would allow the diffusion of the growth factors and waste products out of the graft and cell nutrients into the matrix meanwhile preventing the host immune cells from entering it. Matrices like Matrigel[8] and collagen[54 and 55] have been used in embedding Schwann cells and fibroblasts modified to express NGF respectively and then transplanting them in the spinal cord lesion. Some other materials that have been used to encapsulate these genetically engineered fibroblasts include acrylonitrile-vinyl[56] chloride and polyethersulfone[57] hollow fibers that are then transplanted in the spinal cord. Polypropylene fibers with fibroblasts genetically modified to express CNTF when transplanted subcutaneously in mice resulted in slowing down progressive motor neuropathy[58]. Above mentioned studies show that encapsulating and grafting

genetically engineered cells can aid in neuron survival and growth following injury. They also provide the means by which cells that are genetically modified to express different neurotrophic factors can be encapsulated together in matrices and even encapsulating antibodies to growth inhibitors or a combination of both. The success of this kind of therapy however would depend on cell survival within the matrix and the expression of the desired neurotrophic factors or antibodies to growth inhibitors into the lesion both *in vitro* and *in vivo*. The matrix should also provide the necessary protection for these modified cells from the host immune system. The matrix should also be made from materials that are non toxic to the host. A material that would fulfill the above criteria would be alginate and it also uses mild conditions for encapsulation.

2.3 Alginate

Alginate is a linear polysaccharide derived from brown seaweed. It is a water soluble copolymer consisting of 2 monomeric units of 1-4 linked α-L-guluronic and β-D-mannuronic acid residues. Alginate is a hydrated polyelectrolyte in solution and occurs in a ribbon conformation[59]. The structure of alginate has been studied in detail using X-ray diffraction and infrared spectroscopy.

The D- mannuronate chain forms a 3-fold-left handed helix with weak

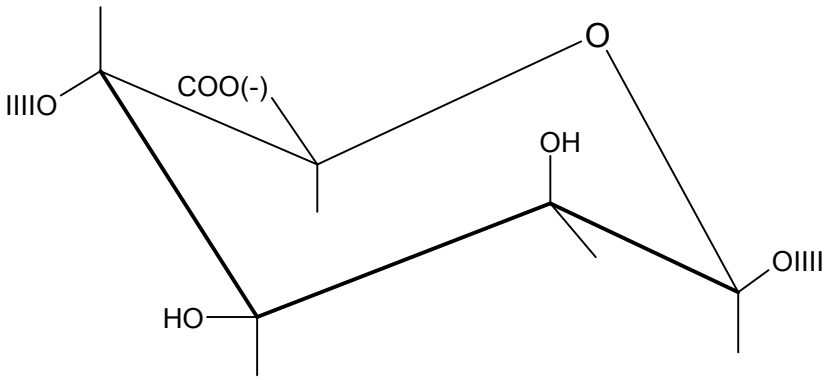
intramolecular hydrogen bonding between the hydroxyl group in position 3 and the following oxygen ring[60]. The L- guluronate on the other hand forms stronger 2-fold screw-helical chains by intramolecular hydrogen bonding between the carboxyl group and the stronger hydroxyl group in position 2 of the previous residues, and the weaker hydroxyl group in position 3. Alternating poly α-(1-4)-linked L-guluronate- β-(1-4)-linked D-mannuronate contains both equatorial-axial and axial-equatorial links and forms a disorderly conformation. They have hydrogen bonds between the carboxyl group on the mannuronate and the hydroxyl groups in the 2 and 3 positions of the subsequent guluronate. The free carboxylic acid groups have a water molecule bound to the carboxylate by a hydrogen bond. These acid residues may be present in varying proportions depending on the source of the alginate; they may be polyguluronic blocks (GG), polymannuronic (MM) or mixed (MG) as shown in table 2.3.1.

Table 2.3.1 Composition of Alginates Obtained from Various Species of Kelp
(from reference 59)

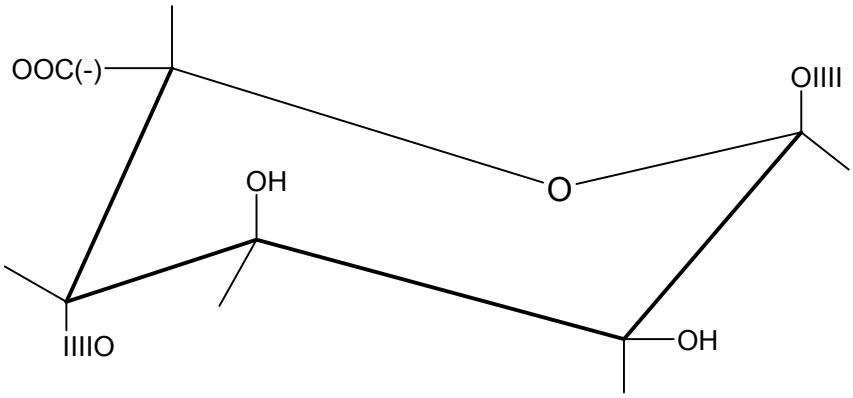
Species	M Content (%)	G Content (%)	M/G Ratio
Ascophyllum nodosum	65	35	1.85
Ecklonia cava	62	38	1.6
Eisenia bicyclis	62	38	1.6
Macrocytis pyrifera	61	39	1.56
Laminaria digitata	59	41	1.45
Laminaria hyperborean	31	69	0.45

Calcium ions can replace the water molecules bound to the carboxyl groups thereby resulting in the closure of the guluronate chains but not the mannuronate, which results in an egg box like conformation[61 and 62]. The chains are stabilized by hydrogen bonding between the other carboxylate oxygen and the hydroxyl group in position 2 of the subsequent residues.

Alginate thus forms gels with multivalent cations like calcium and aluminium and the properties of the gel are governed by the sequence and composition of the monomer chains and the cross linking ion. The greater the content of G monomers



A. Mannuronic Acid



B. Guluronic Acid

Figure 2.3.2: Chemical Structure of the Mannuronic and Guluronic Acid Groups of Alginate (from ref. 59)

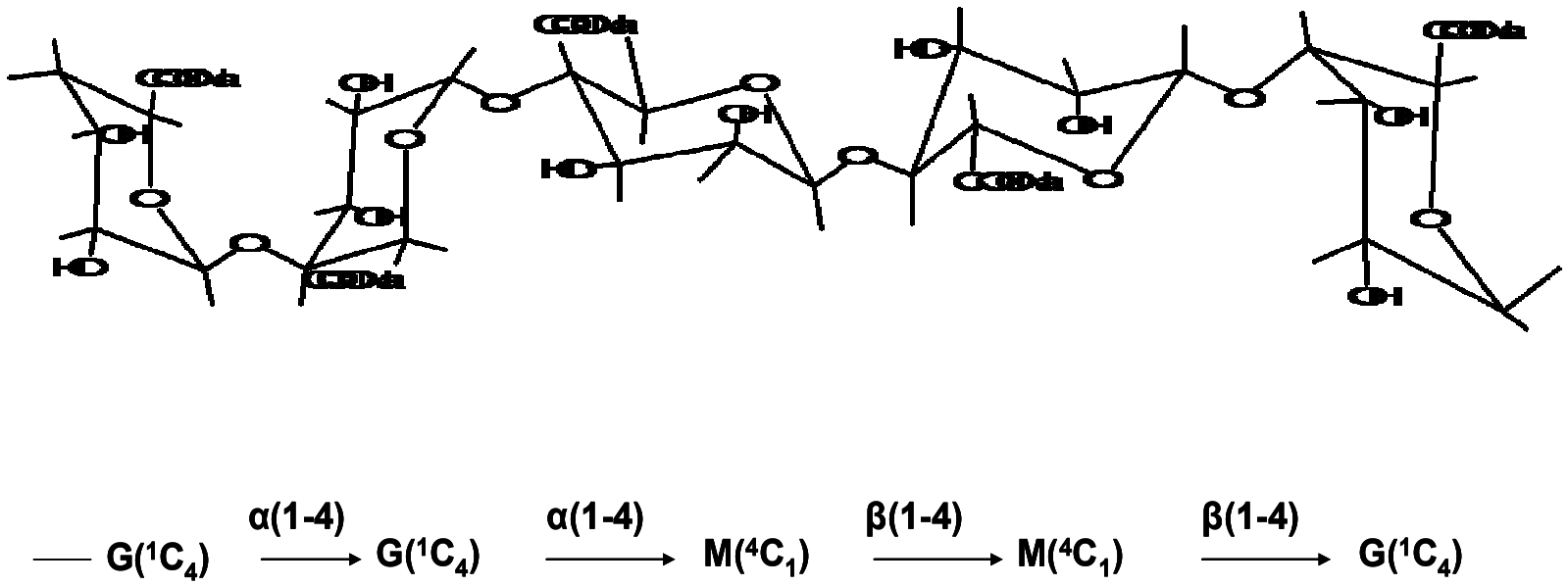


Figure 2.3.3: Chemical Structure of Sodium Alginate (from ref. 83)

the stronger is the gel formed. These gels are similar to alginate. This property of alginate to gel in the presence of a polycation solution (aluminium[63], zinc[64], calcium, and barium[65]) has made it possible for it to be used as a drug delivery agent. The mechanical rigidity of the alginate hydrogels depends on the affinity of the cations to alginate[61]. Studies have shown that the chemical structure, molecular size, and gel forming kinetics of the hydrogel play an important role in its properties including swelling, stability, biodegradability, immunological characteristics, and biocompatibility[35].

Alginate is biocompatible and is harmless to the body[65, 66]. It is used in the food industry as a thickener, emulsifier and as a stabilizer. Alginate belongs to a group of compounds that have been generally regarded as safe by the FDA. It has also been used in tissue engineering for cell encapsulation and various other functions[61]. The pore size of alginate hydrogels formed by crosslinking varies over a wide range. Large proteins like fibrinogen could easily diffuse out of calcium alginate hydrogels in their entirity[67]. Only cells and certain high molecular weight enzymes could be completely immobilized in alginate hydrogels[68] like islet cells, catalaze, etc.

2.3.1 Alginate for Cell Encapsulation

Alginate hydrogels are produced by introducing alginate into an aqueous solution of multivalent cations in and mild environment using non-toxic reactants. Since alginate hydrogels are easy to make and safe they have been studied thoroughly for drug delivery and also believed to be the material of choice for cell encapsulation. The protein release from the hydrogel can be controlled by changing the properties of the hydrogel like varying the concentration of alginate used or changing the crosslinking ion[69]. The rate of release of the drug can also be controlled by coating the hydrogel with polypeptides such as poly-L-ornithine and poly-L-lysine. These polypeptide coatings not only influence the rate of drug release but could also be made to be size selective. In cases where these hydrogels are used for cell encapsulation, the polypeptide coating could act as a barrier against the host immune systems. This can be achieved by varying the molecular weight of the polypeptide in such a manner that it would exclude the immune components but still permit the release of the therapeutic compounds thereby avoiding the need for immune suppressive therapy that would be mandatory following autologous grafts.

Studies have been conducted where pancreatic islets cells have been encapsulated in alginate and transplanted in rats[70]. Following this study various

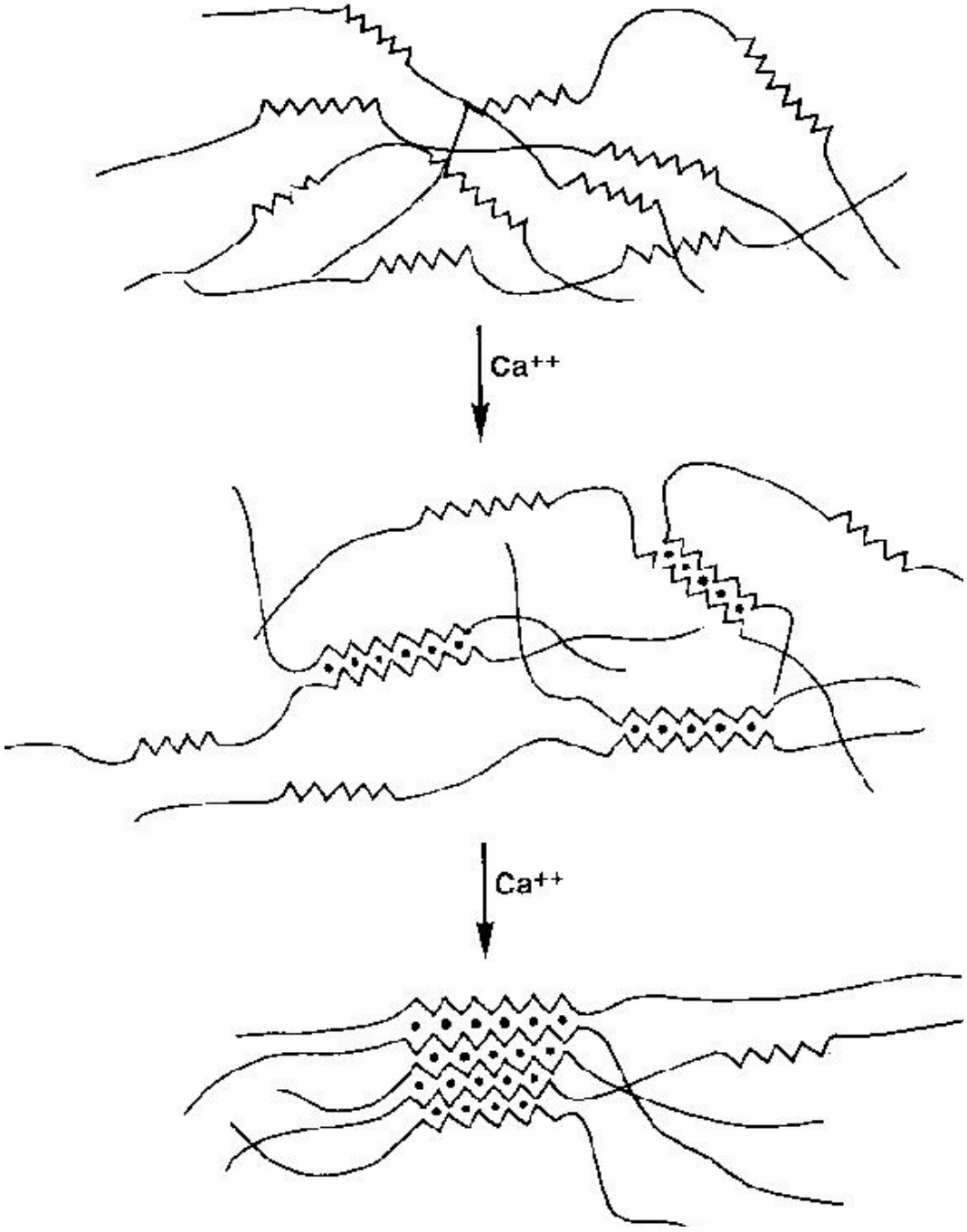


Figure 2.3.4: Alginate Egg Box Model on Cross Linking with Calcium Ions (from ref. 60)

other studies were done in which different cell types like hybridoma cells[71],

hepatocytes[72], renal cells[73] and genetically engineered fibroblasts[9] were used for the delivery of a wide range of compounds like hormones[74 and 75], antibodies[71], angiogenesis inhibitors[76] and neurotrophic factors[9]. Alginate hydrogels have also been used to treat wounds[77] and in tissue repair[78].

2.3.2 Biocompatibility of Alginate

Alginate falls under the category of products that the food and drug administration (FDA) has generally regarded as safe (GRAS). Alginate is currently being used in the food industry as a stabilizer and a thickener. Several studies have also been done where alginate was administered orally to rats and dogs at varying dosages and for different durations and no mortalities or ill effects were observed[59]. *In vitro* studies where alginate has been introduced in cell cultures show that there is very minimal cytotoxicity and alginate is also tolerated in animals when administered orally at a dosage of 500mg/Kg[79]. While most studies show that alginate is non-toxic when used *in vivo* some studies show that the body may produce foreign body reaction to alginate. The foreign body reaction would result in fibrosis around the hydrogel graft which would then be followed by necrosis ultimately reducing or nullifying the effect of the graft[80]. Studies done to elucidate the reasons behind this reaction reveal that the mannuronic acid residues may be

responsible for the foreign body reaction, while other studies show that the guluronic acid residues may induce the necrosis[80]. This type of reaction may be the result of contaminants that are found in crude alginate which was used in the above mentioned study. Alginate can be purified by repeated filtration and precipitation procedures that would help to remove the contaminants and make the alginate biocompatible[81 and 82]. Though the purification helped to prevent fibrosis the lifetime of these grafts ranged from 6 to 20 weeks which was similar to the grafts made of crude alginate. Thus purification of alginate could help in increasing the biocompatibility of the alginate grafts[83 and 84].

2.3.3 Hydrogel Properties and Shapes

The important advantage of using hydrogels for grafts is that they can be made to specific shapes and dimensions according to the need. Alginate has been used in various forms such as hydrogel blocks, spheres, microcapsules, etc. The type, shape and the dimensions of these hydrogels have an effect on the release of the therapeutic compounds entrapped in them. Selection of the appropriate hydrogel scaffold material depends on the physical properties, mass transport property and the biological interactions it would have. These design variables are governed by the particular application they are going to be used for and the environment into which

they would be introduced.

2.3.3.1 Physical Properties

Most scaffolds employed in tissue engineering usually fill a space that is normally occupied by host tissue and act as a framework over which cells could lay down and regenerate into tissue. The mechanism by which gels form dictates the way molecules and cells are incorporated and ultimately their delivery from the scaffold. Once these hydrogel grafts are placed the tissue regeneration would depend on the mechanical properties of the graft. The graft should not only be able to withstand load but also be able to distribute it thereby providing the stability the cells would require to grow on. For alginate these properties would depend on the polymer composition, crosslinking and gelling conditions such as temperature and pH. The mechanical strength and compression of alginate could be increased by increasing the ratio of G to M or by increasing the length of the G blocks[85]. The mechanical strength of ionically crosslinked alginate would increase with the increase in the ion concentration and when divalent cations with higher affinity to alginate are employed[86]. Over time these hydrogels may undergo degradation and dissolution unless the tissue grows into these grafts and helps to strengthen them[87]. The desired rate of degradation of hydrogels depends on the application that it

would be employed for. In tissue engineering however the ideal rate of hydrogel degradation would match the rate of tissue regeneration. Ionically crosslinked alginate usually undergoes dissolution which would depend on the ionic concentration of the environment that it would be place in.

2.3.3.2 Mass Transport Properties

The successes of these hydrogels in tissue engineering applications depends on the appropriate transport of gases, nutrients, cells, waste products, etc. into and out of the scaffolds. Gel properties such as polymer type, polymer size and concentration of the crosslinker ion help to determine the porous nature of the gel[88]. As a result the rate of diffusion into and out of the hydrogels would depend on the size and molecular weight of the particles compared to the pores in the hydrogels. The diffusion of high molecular weight compounds from alginate hydrogels are decreased by increase in the concentration of alginate or increasing the concentration of the crosslinking Ca^{2+} ions. For alginate hydrogels the diffusion of charged molecules would also depend on their interactions with the negatively charged alginate chains[89]. Enhancing the delivery of oxygen and nutrients and removal of waste products is essential for hydrogels with cells immobilized in them. In vivo most cells exist within 100μm of a capillary and diffusion can occur freely

within this distance to ensure cell and tissue survival[13]. So cells need to be immobilized in grafts of small size whereby the cells entrapped are in close proximity to nutrients that would ensure their survival.

2.3.3.3 Biological Properties

Hydrogels that have been designed for grafting in the body must promote desirable cellular functions and tissue functions without eliciting a chronic inflammatory response. As far as alginate hydrogels are concerned the extent of the inflammatory response depends on the purity of the commercially available alginate. Though purified alginate is nontoxic it does not possess any inherent cell adhesive properties. Cells do not have receptors to most hydrogel forming polymers, with the exception of collagen which is an ECM protein, and therefore cells cannot adhere to them. Since hydrogels are hydrophilic, ECM proteins do not easily adsorb onto their surface[90]. The best way to modify hydrogel surfaces to present specific cell adhesive receptors could be achieved by covalently coupling an entire ECM protein[91] or a peptide sequence to the polymer[92 and 93]. The most commonly used peptide sequence for this approach would be the Arginine – Glycine - Aspartic acid - Serine (RGDS)[94] sequence that can be derived from ECM proteins such as laminin, fibronectin, collagen, etc. RGDS peptide however is not specific for nerve cell adhesion. So

other peptides are commonly employed for this purpose which include, Tyrosine – Isoleucine – Glycine – Serine – Arginine (YIGSR from laminin)[95] and Isoleucine – Lysine – Valine –Alanine – Valine (IKVAV also from laminin)[96]. Various studies have been done to prove that the above mentioned peptides could be covalently coupled with alginate and have been found to promote nerve cell adhesion. Peptide modification helps to create analogs of protein modified surfaces also allowing the control of concentration and orientation of the peptides on the hydrogel surface. Studies have shown that YIGSR and IKVAV promote a greater number of neurites per cell[97]. Experiments have also shown that YIGSR and IKVAV modified surfaces selectively promote neuronal cell adhesion[98].

The hydrogels can also be molded to different shapes according to the application. Alginate hydrogels have been molded into different shapes like beads, blocks, films, fibers, etc. for various applications. Macro and micro alginate beads have been employed in delivery of therapeutic drugs such as insulin[99], melatonin[64], etc. Alginate beads also happen to be the vehicle of choice for grafting live cells that would have a therapeutic effect. Some of the common cell lines that have been immobilized in alginate beads are islet cells[65], hepatocytes[73], genetically engineered fibroblasts[9], etc. Alginate blocks with TNF-β1 incorporated in them have been

studied for treating intracranial aneurysms[100]. Alginate films have been tested for use as biodegradable packaging film for disposing waste products[101]. Alginate films have also been studied for delivery of therapeutic drugs like acetaminophen[102]. Alginate fibers can be produced by extruding alginate solution into a calcium ion bath and then letting the fibers dry. These fibers could then be collected on spools for knitted fabrics or chopped for use in non-woven materials. Several commercially available wound dressings have calcium alginate fibers[103]. The in-situ gelling property of alginate has also been utilized for certain applications like prevention of adhesions during tissue repair, treatment of burns, in arresting hemorrhage and ophthalmic drug delivery[104]. An oral controlled release system has also been studied wherein a dry mixture of sodium alginate, calcium phosphite and a soluble drug are compressed into tablets. When administered orally the tablet is acted upon by gastric juices following which the calcium ions interact with the alginate forming a sponge like matrix and the drug release would be dependent on the dissolution of the gel and diffusion of the drug from the gel[105].

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

Manugel DMB a high guluronic acid alginate was a generous gift from ISP Alginates, San Diego CA (formerly the Nutrasweet Kelco Company) was used as obtained. N-[2-hydroxyethylpiperazine-N' –[2-ethanesulfonic acid] sodium salt (HEPES), morpholinoethanesulfonic acid sodium salt (MES), poly-L-ornithine (PLO, MW 15,000-30,000), calcium chloride, sodium chloride, calcium sulfate, sodium metaphosphate, 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide (EDC) and N-hydroxysulfosuccinimide sodium salt (Sulfo-NHS) were purchased from Sigma Chemical Co., St. Louis, MO. The pentapeptide Tyrosine-Isoleucine-Glycine-Serine-Arginine was obtained from American Peptide Company (Sunnyvale, CA). Natural mouse Laminin was purchased from Invitrogen, Carlsbad, CA.

3.1.2 Cells and Cell Culture Products

Rat abdominal skin fibroblasts that have been genetically engineered to release brain derived nerve growth factor (BDNF) were kindly provided by Dr. Itzhak Fisher, Drexel University College of Medicine. The mouse (NB2a) and human (SHSY5Y) neuroblastoma cells were kindly provided by Dr. Tom Shea, University of Massachusetts, MA (Lowell Campus). Fertilized white leg horn eggs

were purchased from Charles River Laboratories Inc., Wilmington, MA. Dulbeco's modification of Eagles medium with glucose (DMEM), Ham's F-12 medium, 0.25% Trypsin EDTA, L-glutamine and Hank's balanced salt solution from Fisher Scientific, (Fair Lawn, NJ). Antibiotics (Penicillin 100IU/ml, Streptomycin 50μg/ml), Dibutryl cyclic adenosine monophosphate (DbcAMP), Retinoic acid (RA), X- Gal Staining Kit, 0.4% Trypan blue and methylene blue were purchased from Sigma Chemical Co., St. Louis, MO. Fetal calf serum (FCS), and Serum Replacement (SR – Knockout) from Gibco BRL, Rockville, MD. 100mm Polystyrene culture plates and 6 well culture plates were from Falcon, Becton Dickinson Labware, Franklin Lakes, NJ.

3.2 Alginate Strings

The 1% (w/v) modified alginate solution was prepared in DI water and sterilized by filter through a 0.45μm Millipore filter. The solution was loaded in a sterile 5 ml syringe with a non-beveled 22G needle. The syringe was mounted on a Sage syringe pump (Orion Research Inc., Beverly, MA model 355) which was wiped down with alcohol and placed under UV for 15 minutes in a plasma hood, and the flow rate was set at 13.5ml/min or a rate of 63ml/min and diameter set at

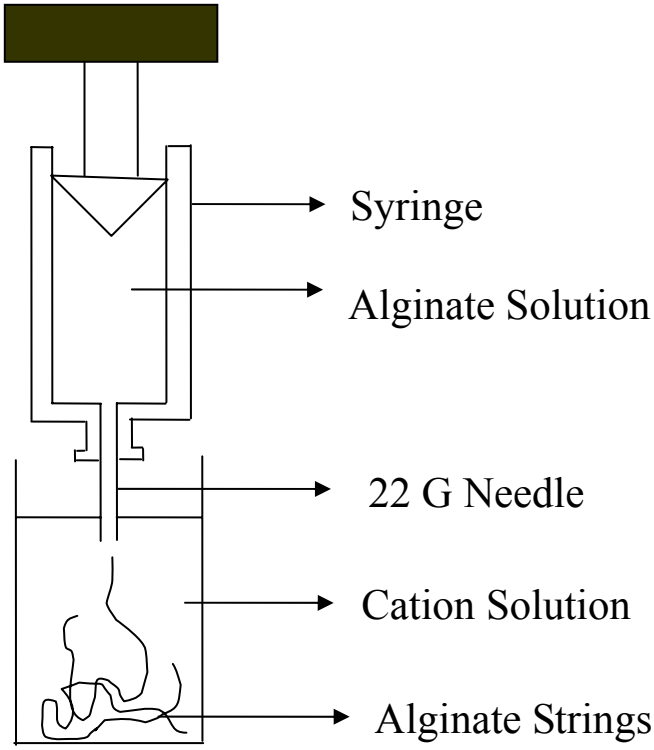
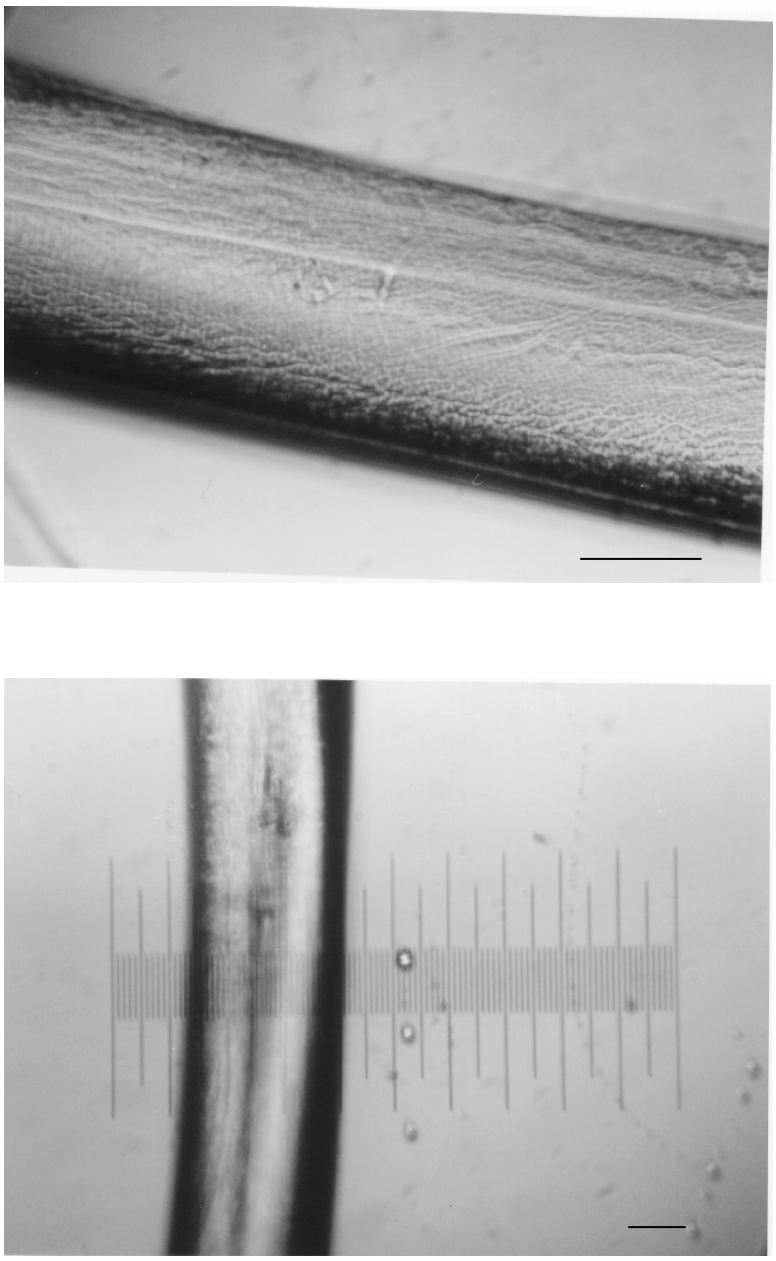


Figure 3.2.1 Schematic of Setup for Preparing Alginate Strings

44mm with the Kent Genie syringe pump. The tip of the needle was dipped in a tall 500 ml beaker containing a 1.3% $CaCl_2$ solution. Calcium cross-linked alginate strings were produced that are allowed to sit in the $CaCl_2$ solution for an hour for hardening and were then washed thrice with 200 ml of HEPES buffer (pH 7.35) per wash. A schematic of the preparation of alginate strings is shown in Figure 3.2.1. The strings were uniform and about 350-450μm in thickness as measured using the micrometer scale shown in Figures 3.2.2a and b.

3.2.1 Coating Alginate Strings

The strings were then coated with poly-L-ornithine, molecular weight 15,000 - 30,000, at a concentration of 0.5mg/ml of alginate for 6 minutes under gentle shaking. The volume of PLO solution used was 6 times the volume of the alginate used for making the strings. The PLO solution was prepared in HEPES buffer just before its addition and was filtered using a 0.2μm sterile cellulose acetate filter into an autoclaved beaker. The strings were then washed 3 times with 200 ml of HEPES buffer to remove any unreacted PLO. The strings were then exposed with 100ml of 1% (w/v) modified alginate solution for 15 minutes. The strings were then washed thrice with HEPES buffer to remove excess unreacted modified alginate. All buffer solutions and glassware used in the experiments were sterilized by autoclaving.



Figures 3.2.2a & b: Alginate Strings

Figure a. is a light micrograph taken at a magnification of 250X shows a piece of alginate string with smooth regular borders. The black like represents a scale of 100μm. Figure b. is a light micrograph taken at a magnification of 100X showing a piece of alginate string on a micrometer scale. The black line represents a scale of 100μm**.**

3.3 Ensheathing Genetically Engineered Fibroblasts

3.3.1 Culture of Genetically Engineered Fibroblasts

Adult Sprague-Dawley rats' fibroblasts that have been genetically modified by a recombinant retrovirus to secrete BDNF (Fb/BDNF) were kindly provided by Dr. Itzhak Fisher, Drexel University College of Medicine. The retroviral vector has the human BDNF transgene linked to a reporter gene LacZ, which coded for the bacterial enzyme β- galactosidase[2].

Fb/BDNF were cultured in10cm tissue culture plates in DMEM supplemented with 10% FBS at 37°C and 5% CO_2. The cells were passaged when they attained 70-80% confluency and it has been shown that the cells express the transgene to a good level upto about 25 passages.

3.3.2 Ensheathing Fb/BDNF in Alginate Strings

Cells were harvested at about 80% confluency using 0.25% (w/v) Trypsin (3ml/plate for 3 minutes incubated at 37C) which helps to break the cells off the surface of the culture plate by enzymatic action. Once the cells were removed they were suspended in 0.9% sterile saline solution. A stock solution of 1.5% (w/v) alginate (Manugel DMB) was sterilized using a 0.45μm bottle top filter. The cells that were suspended in sterile saline were added to the sterile alginate solution to

obtain a 1% (w/v) alginate solution with a cell concentration of about 3 x 10^6 cells/ml of alginate solution. Alginate strings containing the Fb/BDNF were then prepared by the same method as explained in section 3.2. All needles beakers and solutions used were sterilized by autoclaving at a temperature of 132C for 30 minutes in a Brinkmann autoclave. Once the strings were made they were coated using the procedure described in section 3.2.2. The entire procedure involved in making and coating the alginate strings was performed under a laminar flow hood observing strict sterile policies. The strings were then transferred to 10cm sterile culture plates containing fibroblast growth medium and placed in an incubator at 37°C under 5% CO_2.

3.4 *In vitro* Evaluation of Fb/BDNF in Alginate Strings

3.4.1 X-Gal Staining for Ensheathed Cells *In vitro*

After strings with Fb/BDNF have been in culture for 7 days, they were evaluated for the expression of the β-galactosidase reporter gene using X-Gal histochemistry[105]. Strings made from 1ml of alginate containing Fb/BDNF were fixed with 0.5% (w/v) gluteraldehyde solution (1ml/ ml of alginate strings) for 10 minutes and washed thrice with PBS. The strings were then incubated with X-Gal reagent (5-bromo-4chloro-3-indolyl β-D-galactopyranoside) at a

concentration of 1mg/mland X-Gal mixer in PBS (2ml/well) overnight at 37°C. The X-Gal mixer is made up of 35mM $K_3Fe(CN)_6$, 35mM. $K_4Fe(CN)_6.3H_2o$, 2mM Mg Cl_2. The following day the strings in the culture plate were observed using a light microscope and photographs were taken using an Olympus DP-11 digital camera.

3.4.2 Freezing and Thawing Alginate Strings with Fb/BDNF

Strings containing Fb/ BDNF made from equal volumes of alginate were placed in cryovials containing freezing medium (60%DMEM + 30% FBS + 10% dimethyl sulfoxide). The vials were placed in a time delayed freezing container (Nalgene, Fisher Scientific, Fair Lawn, NJ). under -85°C for 24 hours and then transferred to liquid nitrogen. Strings with the Fb/BDNF that were frozen were removed at different time intervals and thawed by placing them in a 37°C water bath. The thawed strings were then transferred to a 10cm culture plates and resuspended in 10ml of pre-warmed fibroblast growth medium and incubated at 37°C for 2-4 hours. The medium was again replaced to remove dimethyl sulfoxide (DMSO). The strings were then dissolved in 10% (w/v) ethylene diamine tetra acetic acid (EDTA) and the cells were resuspended in fibroblast growth medium and pelleted by centrifugation at 1000 rpm's for 5 minutes. The cells were stained using methylene

blue (nucleic acid stain) and the viable cells were counted using a haemocytometer, to verify cell survival following prolonged freezing. The cells were also plated in culture plates with medium to verify growth and stained with X-Gal staining kit to verify the expression of β-galactosidase reporter gene.

3.4.3 BDNF Immunoassay

Strings were made from equal volumes of alginate containing similar Fb/BDNF cell concentrations/ml of alginate. The strings are suspended in Fb growth medium in 12 well culture plates. After 24 hours the medium is removed and an enzyme-linked immunosorbent assay (ELISA) is performed to verify the concentration of the BDNF that is released from the Fb/BDNF[106]. The assay consists of a microplate pre-coated with a monoclonal antibody specific for BDNF. Standards, samples and controls were pipetted into the wells and any BDNF present would then be bound by the immobilized antibody. An enzyme linked monoclonal antibody specific for BDNF was then added to the wells, which was followed by a wash to remove any unbound antibody-enzyme reagent. A substrate solution was then added to the wells and color develops in proportion to the amount of BDNF bound in the initial step. The intensity of the color produced was then read at 450nm using a spectrophotometer and compared to the standard to calculate the

concentration of BDNF present.

3.5 Laminin Conjugated Alginate as a Growth Permissive Surface

3.5.1 YIGSR-Alginate Conjugate Preparation

YIGSR a pentapeptide of laminin is obtained from American Peptide Company. The peptide is bound to the alginate covalently through an amide bond formation between the carboxyl groups of the alginate and the amine terminus of the peptide. This reaction is aided by a zero length crosslinker, 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC). Studies have shown that the amount of EDC present in the reaction has a dramatic effect on the efficiency of peptide incorporation. The EDC is also most reactive in an acidic pH of about 4.5 but a more neutral pH of 6.5 was necessary for peptide incorporation. A detailed description of the method is provided in the Appendix. Different peptide concentration and varying concentrations of the alginate carboxyl groups were activated to compare cell adhesion. Also entire laminin fragment was bound to the alginate using the same procedure and results compared.

3.5.2 NB2a and SHSY5Y Cell Culture

NB2a and SHSY5Y are mouse and human neuroblastoma cell lines, respectively and were obtained as a gift from Prof. Shea (University of

Massachusetts, Lowell Campus). Both cell lines were cultured in 10cm culture plates with DMEM (without L-Glutamine) + 10%FBS + 2mM L-Glutamine + 2mM Antibiotics. The cells were passaged at approximately 70-80% confluency.

3.5.3 Preparation of Modified Alginate Strings and Discs

Modified alginate strings were prepared using the same procedure as that used to make regular alginate strings. Once the strings are coated with PLO they are then coated with 1% (w/v) solution of the peptide modified alginate. NB2a and SHSY5Y cell adhesion and differentiation experiments were carried out using the modified alginate strings. Since the diameter of the chick embryo dorsal root ganglia (DRG) is greater than the width of the strings, modified alginate discs were prepared to verify DRG adhesion and neurite extension.

Modified alginate discs were prepared using calcium sulfate ($CaSO_4$) slurry[91]. A 1% (w/v) solution of modified or unmodified alginate was prepared in deionized water comprising 0.2% (w/v) of sodium metaphosphate which has a minimal effect on reaction efficiency. The addition of $CaSO_4$ to alginate dissolved in DI water lead to formation of precipitates within the disc which could be avoided by the addition dissolving the alginate in sodium metaphosphate. The formation of precipitates may be due to the higher ionic strength of $CaSO_4$ as compared to $CaCl_2$ which could be

countered by the presence of sodium metaphosphate. The solution was sterilized by filtering through a 0.45μm syringe filter. The calcium sulfate slurry was prepared in deionized water at a concentration of 0.4g/ml and sterilized by autoclaving. The required volume of alginate was transferred into a 50 ml centrifuge tube to which the calcium sulfate slurry was added at a concentration of 20μl/ml of alginate. The solution was then shaken vigorously before being poured out into the wells of a 6 well culture plate and allowed to set for an hour. This was carried out in a laminar flow hood to keep the discs sterile and they were then ready for cell adhesion studies.

3.5.4 Cell Adhesion to Peptide Modified Alginate

NB2a and SHSY5Y cell adhesion to peptide modified alginate was studied and compared using varying concentrations of peptide, varying the peptide bound to the alginate and also by varying the concentration of carboxyl groups in alginate that were activated. Modified alginate strings made from equal volumes of alginate were placed in each well of a 12 well culture plate. NB2a and SHSY5Y cells were harvested from culture using 0.25% trypsin, centrifuged , counted and resuspended in serum free medium made up of DMEM + 10% serum replacement + 2mM L-Glutamine. The strings were seeded with 500,000 cells per well and incubated at 37°C at 5% CO_2 for 24 hours. The strings were washed thoroughly with Hank's

buffered salt solution to remove any unattached cells. The strings were transferred to wells of a new 12 well culture plate and treated with 0.25% trypsin 3ml for 5 minutes and incubated at 37°C to detach the cells from the alginate strings and resuspended in growth medium. The cell were centrifuged, stained and counted using a hemocytometer. The statistical significance of cell adhesion to the alginate strings was calculated using Prism 3.0 statistics software package.

This experiment was repeated where the concentration of the peptide added was varied and also a comparative cell adhesion study was performed using YIGSR and laminin at varying concentrations. Cell adhesion studies were also conducted by varying the concentration of carboxyl groups of alginate that were activated. Unmodified plain alginate strings were employed in all the above experiments to act as controls. The protocol for counting cells using a haemocytometer is provided in the appendix.

3.5.5 Cell Differentiation on YIGSR Modified Alginate Strings

The neuroblastoma cell lines were also studied for their ability to extend neurites along the alginate strings to verify if these strings could act as guidance channels. Alginate strings were made from equal volumes of YIGSR modified alginate and placed in 12 well culture plates as explained in section 3.5.4. After 24

hours when the strings were washed with Hanks salt solution to remove any unattached cells, the strings were resuspended in prewarmed specific differentiation medium. For NB2a cells the differentiation medium consists of serum free medium + 0.1% FBS + 10μM Dibutryl cyclic adenosine monophosphate (DbcAMP) and for the SHSY5Y cells the differentiation medium includes serum free medium + 0.1% FBS + 5μM Retinoic acid (RA). The differentiation medium was replaced everyday to replenish the DbcAMP and RA respectively. Cell differentiation and neurite extension were observed for a week using a microscope and pictures were taken periodically using a DP-11 digital camera (Olympus Optical Co. Ltd., Japan) mounted on the microscope.

3.5.6 Chick Embryo Dorsal Root Ganglion Extraction

Fertilized white Leghorn eggs were obtained from Charles River Laboratories Inc., Wilmington, MA. The eggs were placed in an incubator with an automatic turner at 39°C which is the ideal temperature recommended for embryo growth. Eggs were removed from the incubator between days 8 and 10 for dissection. Eggs were sprayed with alcohol before their shells were cracked open and embryos extracted. The embryos were place in sterile culture plates and dissected using dissecting forceps under a dissecting microscope. The thoracic cavity of the embryo

was split open following which the organs were removed piece meal to expose the spine. The DRGs were then collected from either side of the lumber vertebrae and placed in a culture plate with Hams F-12 medium. Aforementioned steps were carried out under aseptic conditions.

3.5.7 DRG Adhesion and Growth on Laminin Modified Alginate

Once the dissections were completed the DRGs were placed on laminin modified alginate discs. The DRGs were allowed to sit on the laminin modified discs in 6 well culture plates for 15 minutes to ensure adhesion before medium was added to it. The DRGs on 3 discs were exposed to 1ml fibroblast growth medium each; DRGs on 3 discs were exposed to 1ml fibroblast growth medium each that was removed from three wells of a 12 well culture plate containing modified alginate strings with 10^6 Fb/BDNF (~ 10ng of BDNF). DRGs on 3 other discs were exposed to 1ml of fibroblast growth medium supplemented with 10ng of human BDNF (Sigma) each. DRG adhesion and neurite extension on the laminin modified alginate discs was observed for 48 hours and images were captured at 24 and 48 hours using an inverted Nikon microscope model TE2000 and phase contrast pictures were also captured using an Olympus camera mounted on an inverted light microscope. The length of the neurites was measured using Northern Eclipse

software. Thirty randomly chosen neurites were measured from each DRG and plotted on a graph and statistical analysis of one way ANOVA was performed using Graph Pad software.

3.6 Efficiency of PLO Coating on Immunoglobulin Exclusion

The alginate strings were coated with poly-L-ornithine of molecular weight of 15,000 – 30,000. This would help to prevent host immune cells from entering the graft and acting on the ensheathed Fb/BDNF. The efficiency of this PLO coating could be verified by exposing the modified alginate strings containing the Fb/BDNF to a cell specific antibody. The cells would then be removed from the strings and treated with a fluorescent secondary antibody. The cells would only take up the secondary antibody if the primary antibody had managed to get on the cells eluding the PLO coating. The antibody chosen for this experiment was the IgG antibody to the peroxisomal membrane protein 70 (PMP 70) (molecular weight 70kDa), which is a high-abundance integral-membrane component of peroxisomes[108 and 109]. The molecular weight of the most common antibodies found in the body ranges from 65 to 150kDa. Peroxisomes are single membrane-bound vesicles that are found in all animal cells except red blood corpuscles (RBC's). The function of peroxisomes is enzymatic oxidation of fatty acids and catalyzes the breakdown of hydrogen

peroxide (H_2O_2) that is a byproduct of fatty acid degradation. Alexa Flour 488 peroxisome labeling kit which is a fluorescent antibody labeling kit was obtained from Molecular Probes (Eugene, OR).

The Fb/BDNF ensheathed in alginate strings with and without PLO coating, that were freshly made and those that were frozen and thawed, were exposed to the anti-PMP 70 antibody. The strings were incubated at 37°C for 2 hours. The cells were then isolated from the strings by dissolving them in sterile 10% (w/v) EDTA solution. The cells were plated on cover slips, fixed with methanol free 16% (w/v) formaldehyde solution and exposed to the Alexa Fluor 488 fluorescent secondary antibody and incubated for 30 minutes at 37°C. The secondary antibody would bind only to the cells that have the primary antibody on them. The cells were then viewed using an Eclipse TE 2000U inverted fluorescent microscope (Nikon, Melville, NY) and pictures were taken using a Cool Snap Photometrics camera (Roper Scientific, Tucson, AZ). This experimental setup would help to determine if the PLO coating is effective in keeping the IgG immunoglobulins from entering the alginate strings and affecting the Fb/ BDNF and help to verify if the strings could be grafted without immune suppressive therapy.

4. RESULTS AND DISCUSSION

In this thesis we developed a graft made of alginate that would have applications in aiding spinal cord regeneration. We hypothesized that modifying the alginate with laminin would aid in neuronal cell adhesion and growth. We also hypothesized that ensheathing genetically engineered fibroblasts in the graft would not only provide growth factors for neurons but would also avoid immune suppression that would be required.

Grafts were designed and optimized in the form of alginate strings by varying the type of alginate; needle size and syringe pump speed. The graft was hen further optimized by modifying it with laminin which is a component of the basement membrane and would aid in neuronal cell adhesion. The ideal conditions were optimized by using varying laminin concentration and also varying the moles of carboxyl groups involved. Genetically engineered fibroblasts that produce BDNF were ensheathed in the alginate strings and these strings were then studied *in vitro* for their effects on neuronal cell adhesion and growth. Lastly the strings were also frozen and thawed at specific time intervals to verify if this graft could be stored and made readily available for grafting soon after injury.

4.1 Cell Adhesion to YIGSR Modified Alginate Strings

Modified alginate strings were produced as described in Appendix 2 under sterile conditions. Modified alginate strings made from 1ml of alginate were placed in 6 wells of a 12 well culture plate. The remaining 6 wells contained strings made from 1 ml of plain alginate Another set of controls was prepared with 6 wells containing strings made from 1 ml of plain alginate and coated with PLO to examine the effect of the PLO coating had on cell adhesion. Two different neuroblastoma cell lines were used to estimate the cell adhesion to the modified alginate strings. The NB2a cells are the rat neuroblastoma cells while the SHSY5Y cells are human neuroblastoma cells. These cells are also model cell lines derived from neuroblastomas and would not exactly mimic the neurons *in vivo*. These cells were undifferentiated and required specific differentiating factors to extend neurites. The neuronal cells in the body would however be differentiated cells and results may not be similar to that of the undifferentiated cells in the presence of differentiating factors. The NB2a cells grow more confluently than the SHSY5Y cells. The cells were added at a concentration of $5*10^5$ cells per well to 3 wells each of the modified alginate strings, plain alginate strings and plain alginate strings coated with PLO. One ml of plating medium (DMEM with 2mM L-glutamine +

10% serum replacement +2mM antibiotic solution) was added to each of the wells. The serum replacement lacks fibronectin that is found in fetal bovine serum and would not affect the cell adhesion. This would help to ensure that the cell adhesion was solely influenced by the peptide bound to the alginate and not by fibronectin present in serum. The culture plates were incubated at 37°C at 5% CO_2 for 24 hours following which pictures were taken under the microscope and the strings were removed with sterile forceps and placed in wells of a fresh culture plate and washed with Hanks buffered salt solution thoroughly. The strings were then trypsinized and resuspended in growth medium and the medium was removed and centrifuged. The cells were then stained with 0.4% trypan blue and counted using a haemocytometer.

Figure 4.1.1 shows the results of the cell adhesion to the different types of strings made from 1ml of alginate. The results show that the SHSY5Y cell adhesion to the modified alginate strings is about 26.4% of the initial concentration of cells added and the NB2a cell adhesion to the modified alginate strings is about 34.8% of the initial concentration of cells added. Since the strings are in serum free medium the cells would not have enough nutrients for significant replication. Both sets of controls show no significant cell adhesion to either cell type. The above cells show that the NB2a cells show significantly better adhesion ($p<0.01$) to the modified

alginate strings than the SHSY5Y cells. Figure 4.1.2a and b show NB2a and SHSY5Y cell adhesion to modified alginate strings. The NB2a cells are more confluent in culture and robust when compared to the SHSY5Y cells, which might be the reason for their better adhesion to the modified alginate strings. These are light micrographs take 24 hours after seeding at a magnification of 250X. This proves that strings made from alginate modified with YIGSR do promote neuronal cell adhesion. Plain alginate has no intrinsic cell adhesive properties and PLO by itself does not affect cell adhesion.

4.1.1 Cell Adhesion with Increasing Peptide Concentration

Previous experiments done by Dr. Dhoot in our lab showed that cell adhesion on modified alginate discs improved with increasing peptide concentration. NB2a cells were used in all subsequent experiments since they were confluent in culture and easier to handle. The initial concentration of peptide used to modify the alginate was 1mg/gm of alginate based on previous work. The concentration of peptide was varied, ranging from 1mg/gm of alginate to 4mg/gm of alginate. An experimental setup consisted of a 12 well culture plate containing 3 wells each of modified alginate strings made from 1 ml of alginate with varying YIGSR concentrations. NB2a cells were added to each of the wells at a concentration of $5*10^5$ cells per well.

The strings were suspended in plating medium for 24 hours and the strings were then removed washed thoroughly and the cells counted as described earlier. The results are shown in figure 4.1.3.

The results show that there was an increase in NB2a cell adhesion as the concentration of YIGSR increased. Statistical analysis using a one way analysis of variance showed that while there was a significant increase ($p<0.01$) in NB2a cell adhesion between 1 and 2 mgs of YIGSR there was no significant increase ($p>0.5$) in cell adhesion between 2 and 3 mg. These results show that the maximum cell adhesion was achieved with 2 mg of YIGSR/gm of alginate with 0.05 moles of the carboxylate groups activated. This shows that more YIGSR bound to the alginate provides more adhesion sites for the cells thereby improving the percentage of cell adhesion. These results relate to the maximum peptide loading achieved with 0.05 moles of the carboxyl groups on the alginate activated in terms of adhered cells. This indicated that the number of cells adhering to the modified alginate in the form of strings had reached a maximum; with 2mg of YIGSR with 0.05 moles of the carboxylic acid groups were activated. This does not however tell us whether the maximum peptide that could be loaded with 0.05 moles of carboxylic acid groups activated would be 2 mg. The maximum peptide loading on alginate might however

have to be calculated using a high performance liquid chromatography. These results also did not prove if the optimum cell adhesion with modified alginate strings had been achieved.

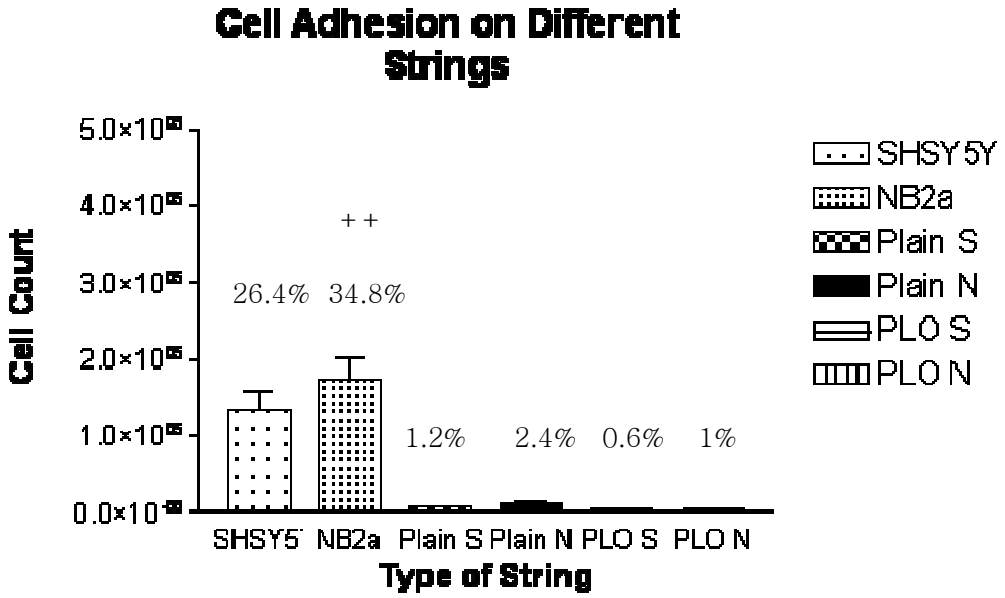
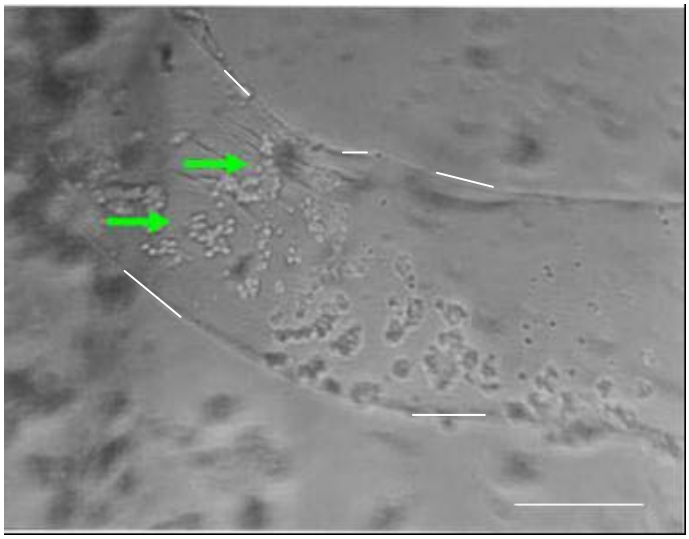


Figure 4.1.1: Neuronal Cell Adhesion to Different Types of Strings.

The graph depicts the amount of NB2a and SHSY5Y cells that attached to modified alginate strings and the plain alginate strings (Controls S and N) and alginate strings coated with just PLO (PLO S & PLO N), after 24 hours. The Y-error bars represent the standard error and n=3. The average cell count of the SHSY5Y cells was 132,000(26.4%) and that of the NB2a cells was 174,070 (34.8%). The average cell count of the SHSY5Y and NB2a cells on the control samples were 5900 and 12,300 respectively. The average SHSY5Y and NB2a cell adhesion to the PLO coated strings were 3140 and 4860 respectively. The values are a percentage of the cells originally added to each well containing the alginate strings. The X axis represents the different string types and the Y axis represents the cell count.

a.



b.

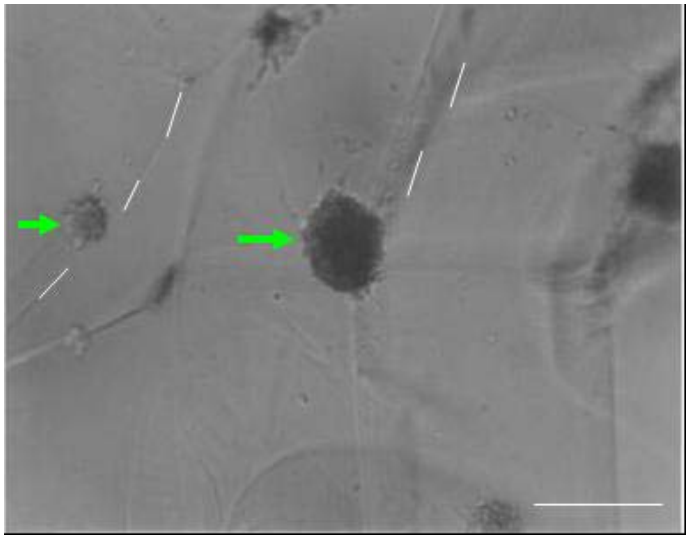


Figure 4.1.2a and b: NB2a and SHSY5Y Cell Adhesion on Modified Alginate Strings

The pictures are light micrographs taken at a magnification of 250X. Figure a. shows NB2a cells on modified alginate strings and b. shows SHSY5Y cells. The dotted lines represent the borders of the strings, the arrows show the cells and the size bar represents 100µm

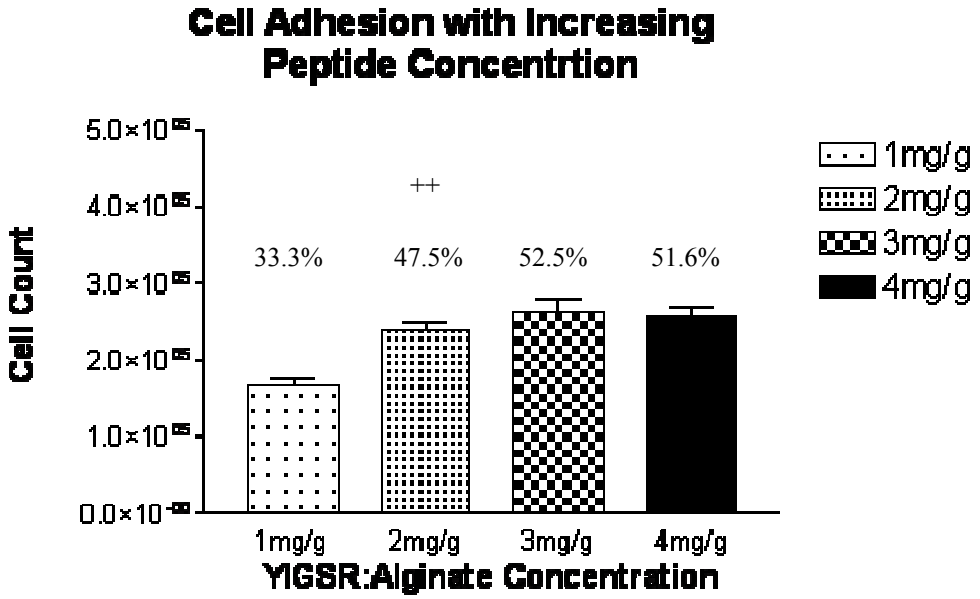


Figure 4.1.3: NB2a Cell Adhesion with Increasing Peptide Concentration

This graph is a quantitative comparison of cell attachment of the NB2a and the cells to modified alginate strings with increasing YIGSR loading per gram of alginate at 24 hours. The Y-error bars represent the standard error. The average cell counts of the NB2a cells to 1mg YIGSR/g of alginate was 166,667 (33.3%), to 2mg/g of alginate was 237,500 (47.5%), to 3mg/g of alginate was 262,500 (52.5%) and to 4mg/g of alginate was 258,333(51.6%), n=3. The increase in cell adhesion between 1 and 2mg YIGSR/g of alginate is statistically significant '++' ($p<0.1$). The X axis represents the YIGSR concentration/g of alginate and the Y axis represents the cell count.

4.1.2 Effect of Surface Peptide Density on Cell Adhesion

To further increase cell adhesion we increased the moles of carboxyl groups of alginate that were activated while varying the concentration of the peptide added. The concentrations of EDC and N-hydroxysulfosuccinimide used in the activation step were varied according to the specific number of moles per gram of carboxyl groups that were to be activated. Equal concentrations of the YIGSR pentapeptide were added to these samples. The moles of hydroxyl groups activated ranged from 0.05 moles to 0.5 moles per gram of alginate. This experiment was then repeated for increasing concentrations of the peptide ranging from 1mg/gm of alginate to 3 mg/gm of alginate. The experimental set up was similar to that described previously. NB2a cells were added at a concentration of $5*10^5$ cells per well to modified strings made from equal volumes (1 ml) of alginate. Figures 4.1.3, 4.1.4 and 4.1.5 show the results of cell adhesion with increasing moles of carboxyl groups activated for different peptide concentrations. The results showed that there was an increase in NB2a cell adhesion with increasing peptide concentration and increasing moles of carboxyl groups being activated. Maximum cell adhesion was achieved with 3mg of YIGSR per gram of alginate with about 0.3 moles of the carboxyl groups of the alginate being activated. These results showed that more peptide could be conjugated to the alginate when more carboxyl groups were activated, in terms of

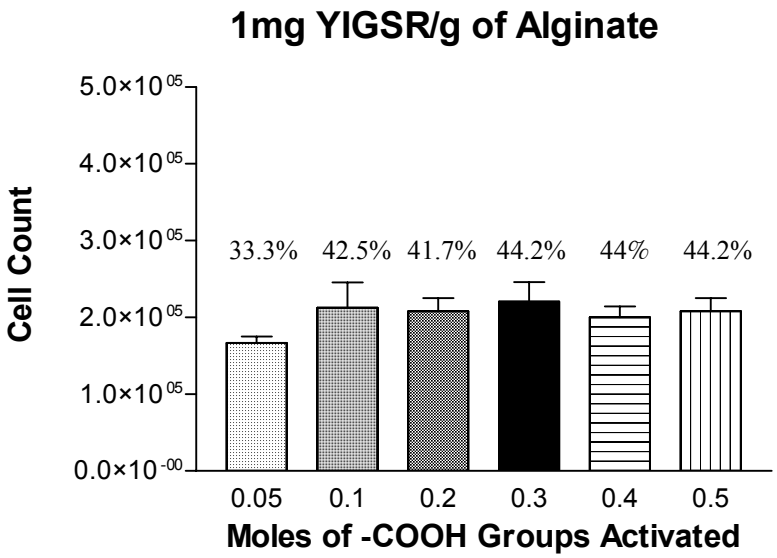


Figure 4.1.4: NB2a Cell Adhesion to Alginate Strings Modified with 1mg YIGSR/gm of Alginate with Varying Concentrations of Carboxyl Groups Activated

This graph is a quantitative comparison of cell attachment of NB2a cells to alginate strings modified with 1mg YIGSR per gram of alginate with varying moles of carboxyl groups being activated. The Y-error bars represent the standard error. The average cell adhesion to strings with 0.05M of –COOH activated was 166,666.7 (33.3%), to 0.1M was 216,666.7 (42.5%), to 0.2M was 208,333.3 (41.7%), to 0.3M was 220,833.3 (44.2%) to 0.4M was 200,000 (44%) and to 0.5M was 208,333.3 (44.2%). There was no statistically significant difference in cell adhesion to the different groups. The X-axis represents the varying moles of –COOH groups in alginate that were activated.

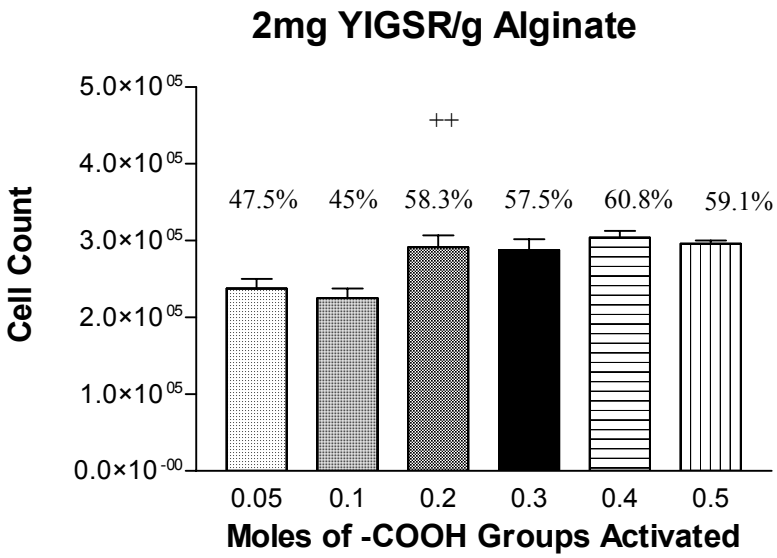


Figure 4.1.5: NB2a Cell Adhesion to Alginate Strings Modified with 2mg YIGSR/gm of Alginate with Varying Concentrations of Carboxyl Groups Activated

This graph is a quantitative comparison of cell attachment of NB2a cells to alginate strings modified with 2mg YIGSR per gram of alginate with varying moles of carboxyl groups being activated. The Y-error bars represent the standard error. The average cell adhesion to strings with 0.05M of –COOH activated was 237,500 (47.5%), to 0.1M was 225,000 (45%), to 0.2M was 291,666.7 (58.3%), to 0.3 M was 287,500 (57.5%), to 0.4M was 304,166.7 (60.8%) and to 0.5M was 295,833.3 (59.1%). There was a statistically significant ($p<0.01$) increase in cell adhesion with the 0.2, 0.3, 0.4 and 0.5 moles of –COOH groups being activated compared to 0.1M group. The X-axis represents the moles of –COOH groups activated and the Y-axis represents the cell count.

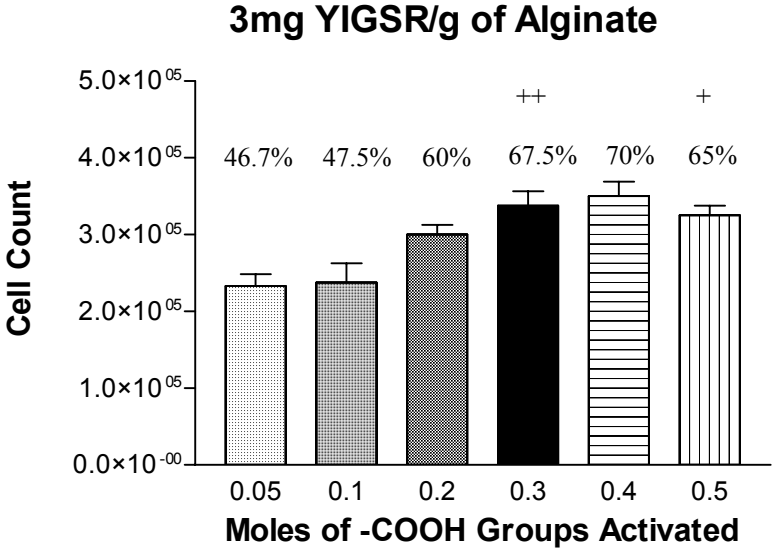


Figure 4.1.6: NB2a Cell Adhesion to Alginate Strings Modified with 3mg YIGSR/gm of Alginate with Varying Concentrations of Carboxyl Groups Activated

This graph is a quantitative comparison of cell attachment of NB2a cells to alginate strings modified with 2mg YIGSR per gram of alginate with varying moles of carboxyl groups being activated. The Y-error bars represent the standard error. The average cell adhesion to strings with 0.05M of –COOH activated was 233,333.3 (46.7%), to 0.1M was 237,500 (47.5%), to 0.2M was 300,000 (60%), to 0.3M was 337,500 (67.5%), to 0.4M was 350,000 (70%) and to 0.5M was 325,000 (65%). There is a statistically significant ($p<0.01$) increase in cell adhesion to the groups where 0.3 and 0.4 moles of –COOH groups were activated and ($p<0.05$) in the 0.5M group as compared to the 0.05M group. The X-axis represents the number of moles of –COOH groups in alginate that were activated. The Y-axis represents the cell count.

cell adhesion. Varying the concentration of the EDC and sulfo-NHS added to the alginate helped to activate a higher number of carboxyl groups. Free carboxyl groups that were not bound to the calcium ions would be available for the YIGSR peptide to bind to. Since alginate has non inherent cell adhesion properties the percentage of cells adhering to the strings would depend on the concentration of peptide bound to the alginate. Higher concentration of peptide would lead to more cell adhesion sites in the available surface area of the graft. These results helped to confirm that the loading of YIGSR peptide to the alginate could be increased by increasing the moles of carboxyl groups that were activated, which was demonstrated by the increase in NB2a cell adhesion. Increasing the peptide loading above 3mg per gm of alginate or further increasing the moles of carboxyl groups activated did not significantly enhance cell adhesion. The reason for this could be that the maximum peptide that could be loaded to alginate had been reached. It could also be due to the fact that the maximum cell adhesion to the graft in the shape of strings had been achieved. Since cells have shown better adhesion and communication on flat surfaces, the cylindrical shape might have an influence on limiting their adhesion. Previous experiments done on cell adhesion on flat surfaces consisting of alginate discs modified with YIGSR have shown better cell adhesion[110].

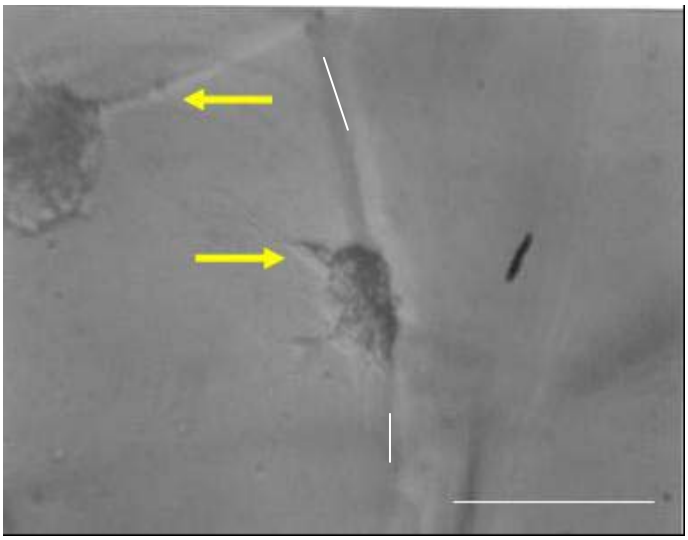
Discs made from alginate modified with 2mg of YIGSR/gm of alginate with 0.05 M of the carboxyl groups have shown 65% - 70% cell adhesion when seeded with NB2a cells. Therefore the cylindrical shape of the graft might indeed play a role in cell adhesion. These results only prove that the optimum cell adhesion to the modified alginate strings had been achieved with a peptide loading of 3mg/gm of alginate with 0.3 to 0.4 M of carboxyl groups being activated. Loading more peptide might also lead to the crowding of adhesion sites which present as a single adhesion site. Also loading of high concentrations of peptide would lead to down regulation of axon growth from the adherent neuronal cells.

4.2 Cell Differentiation on Modified Alginate Strings

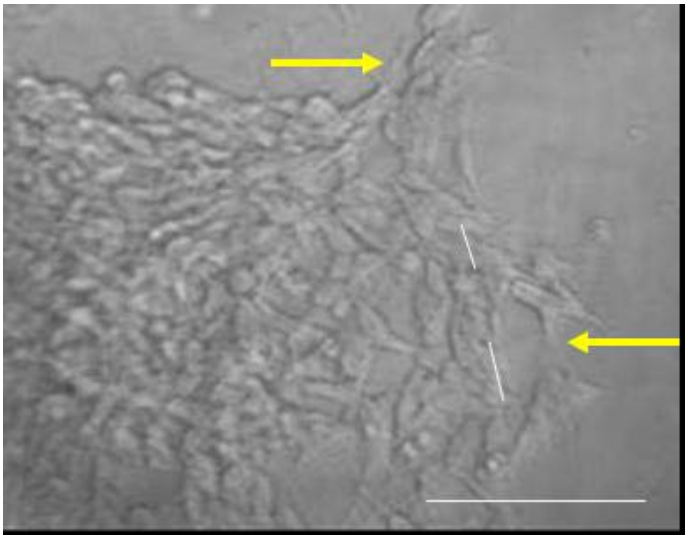
Since cell adhesion had been established by the above experiments the next step was to verify if these cells would extend neurites along the strings thereby showing that these strings could act as a bridge at the injury site. Since the cell lines that we employed were undifferentiated cells specific differentiation factors had to be added once cell adhesion was achieved. The specific differentiation factors for the NB2a and the SHSY5Y cells are Dibutryl cyclic adenosinemonophosphate and retinoic acid respectively. The experimental setup for neuronal cell differentiation was the same as was described in section 3.5.5. The cells were observed for neurite

extension for a week and pictures were taken. Figures 4.2.1a and b show neurite extension from NB2a and SHSY5Y cells respectively, after 2 days in differentiation medium. Figure 4.2.2 shows SHSY5Y differentiation on modified alginate strings after 7 days exposure to differentiation medium. These results show that these cell lines adhered to the modified alginate strings and also extended neurites. The SHSY5Y cell line showed neurite extension along the string indicating that these strings could act as a physical bridge at the injury site. The NB2a cells showed neurite extension but these were short and did not show extensions along the strings similar to the SHSY5Y cells. The modified alginate strings would be the best surface available for the neurons when grafted at the injury site. We can surmise that in vivo, once they lay down on the modified alginate strings the neurons would extend along the strings to communicate with other neurons.

a.



b.



Figures 4.2.1a and b: NB2a and SHSY5Y Cell Differentiation on Modified Alginate Strings at 48 hours

Light micrographs showing NB2a and SHSY5Y cells on modified alginate strings at 250X respectively. Arrows show the neurites extending from the cells. Dotted white line indicates the border of the string which extends from the top of the picture towards the bottom right side. The size bars represent 100μm

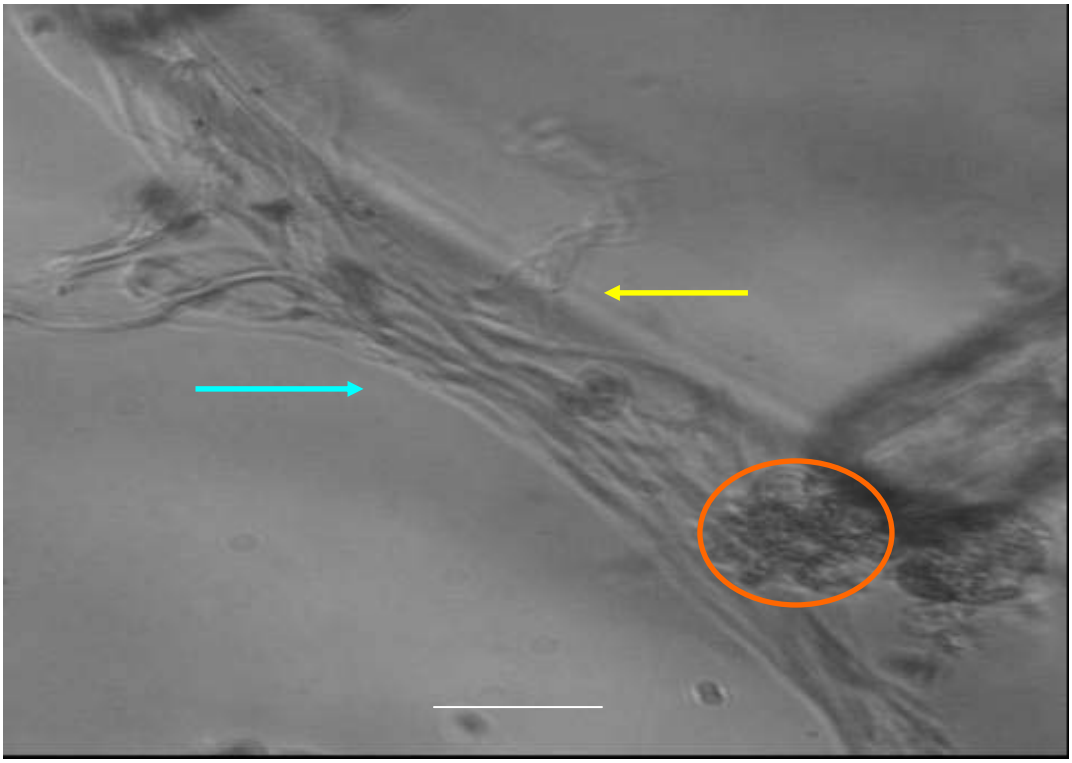


Figure 4.2.2: SHSY5Y Cells on Modified Alginate String at Day 7

A light micrograph taken at a magnification of 625 X showing neurite extensions from SHSY5Y cell bodies on a piece of modified alginate string. The orange circle marks the SHS5Y cell bodies, the blue arrow shows the neurites and the yellow arrow points to the border of the string, which extends from the top left of the picture to the bottom right side. The size bar represents 100μm.

4.3 *In Vitro* Evaluation of Genetically Engineered Fibroblasts Ensheathed in Modified Alginate Strings

Rat abdominal skin fibroblasts that were genetically engineered to produce BDNF were ensheathed in the alginate strings by suspending them in the alginate solution from which the strings were made. This was done so that the graft when applied to the injury would also act as a source of neurotrophic factors at the site. The aim of ensheathing the fibroblasts in the modified alginate strings is to protect the cells from the host immune system while allowing the BDNF to diffuse into the host injury site. The survival and behavior of these ensheathed fibroblasts were then tested *in vitro*. Fibroblast cell survival and growth in modified alginate strings, transgene expression and quantification of BDNF release were investigated.

BDNF producing fibroblasts were obtained from Dr. Fisher's laboratory at Drexel University College of Medicine, where fibroblasts obtained from adult Sprague Dawley rats had been modified using a recombinant retroviral vector (as seen in figure 4.3.1) to produce BDNF. The retroviral construct comprises of the human BDNF transgene linked with a reporter gene which would facilitate the monitoring of the BDNF expression. The reporter gene used in this case was LacZ which codes for the bacterial enzyme β-galactosidase. The long terminal repeats (LTR's) and the internal ribosome entry sites (IRES) in the retroviral vector are

responsible for the expression of the BDNF transgene and the reporter gene via transcription of a polycistronic messenger ribonucleic acid (mRNA). The procedure for making the recombinant retroviral vector and transfection of the fibroblasts with the vector have been described in detail by Liu at al[100]. They also calculated that the rate of BDNF released from these fibroblasts was about 12.8 ng per million cells per 24 hours using the slot blot technique.

4.3.1 Fb/BDNF Growth in Modified Alginate Strings

Fibroblasts were ensheathed in modified alginate strings as described in section 3.3.2. The fibroblasts were suspended in sterile saline at pH 7.4 and added to a 1.5% (w/v) alginate solution to make a 1% (w/v) solution before the strings are made. The strings are then made and coated as descried in sections 3.2.1 and 3.2.2. The cells were loaded at a concentration of $3*10^6$ cells per ml of alginate and strings were placed in the wells of a 12 well culture plate in growth medium. Strings made from 1 ml of alginate were placed in each well and strings from 3 wells were removed at day 7, 14 and 21 and dissolved in EDTA to verify cell survival. The cells were stained with 0.4% methylene blue vital stain. Figures 4.3.2a and b show modified alginate strings with the Fb/BDNF ensheathed in them.

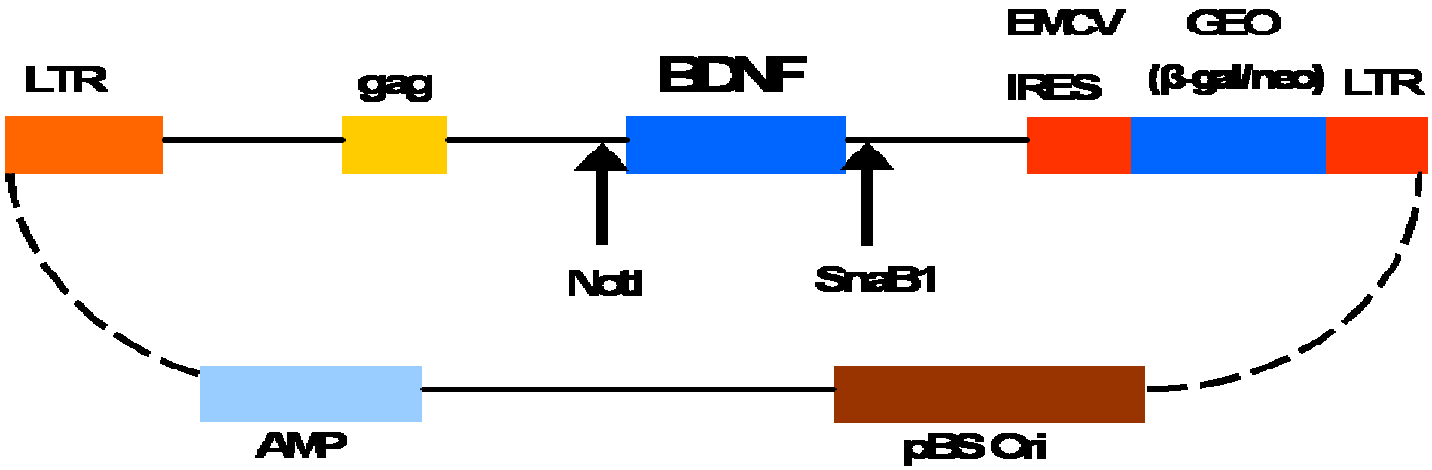


Figure 4.3.1: Structure of the Retroviral Vector

The vector comprises of the human BDNF gene linked with a LacZ reporter gene. The reporter gene helps to monitor the transgene expression and the LTR's drive the expression of the BDNF and the reporter genes.

The cells were found singly and dispersed evenly throughout the strings. Experiments done previously by other researchers, where cells were encapsulated in microcapsules, the cells were found to aggregate in the centre of the capsule forming what were termed “fibrospheres”[76]. No such phenomenon was observed with the modified alginate strings. The results of Fb/BDNF cell survival are shown in figure 4.3.3. The results show that about 93 – 95% of the cells survive upto 3 weeks in growth medium. There is no significant ($p>0.5$) difference in cell survival between the different time intervals. The lack of significant increase in cell count could be due to death of cells that were in the middle of the strings since they would not receive nutrients as opposed to cells that were closer to the surface that would communicate better with the external environment. These cells on the surface might reproduce while the cells closer to the center of the strings probably died due to lack of nutrients. There may have been cell loss while strings were being dissolved in EDTA. Unlike the studies with alginate microcapsules by Dr. Dhoot where encapsulation of higher concentration of cells lead to loss of capsule integrity after 7 days in culture, there were no visible changes in the string integrity and the cells did not aggregate suggesting that the concentration of cells per ml of alginate could be

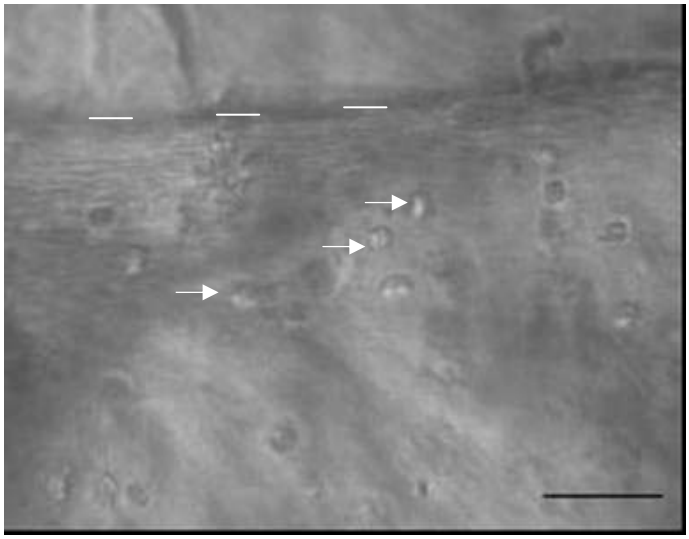
increased. Once cell survival was verified the next step was to verify reporter gene expression and quantify BDNF secretion.

4.3.2 X-Gal Staining of Fb/BDNF in Strings

The alginate strings would be effective in enhancing neuronal regeneration *in vivo* if the Fb/BDNF continued to secrete BDNF for about 3-4 weeks at a concentration of about 20-30ng per 24 hours. Previous experiments, where genetically modified cells were encapsulated in alginate, show that the cells continued to express their active components like growth factors, proteins and other peptides[72, 73, 100, 101]. To verify BDNF expression from the cells ensheathed in the strings they were stained for the β-Galactosidase marker as described in section 3.4.1. Figure 4.2.4a shows staining of the Fb/BDNF for the reporter gene 2 weeks after ensheathing in strings. Figure 4.3.4b shows cells, which were released from the strings by dissolving them in EDTA, staining for the β-Galactosidase marker after 3 days in culture. The reporter gene staining of cells released from the strings was found to be similar to free Fb/BDNF in culture. These results showed that the Fb/BDNF continued to express the BDNF transgene when cultured *in vitro*, 2 weeks after being ensheathed in modified alginate strings. Fb/BDNF that were released from these strings also showed transgene expression upon staining which was

comparable to free Fb/BDNF in culture. This experiment showed that the Fb/BDNF cells were still receiving nutrients from the growth medium to keep them alive and that their transgene expression had not been inhibited by the process of ensheathing them in the strings. Previous *in vivo* work done by Dr. Dhoot, with encapsulated cells in rat spinal injury models, showed that capsules that were harvested 2-4 weeks after transplant showed down regulation of reporter gene activity as compared to that in culture prior to grafting. The harvested cells showed increase in transgene expression when placed in culture for a week suggesting that the *in vivo* down regulation might be the result of soluble factors since the Fb/BDNF had no direct contact with the spinal cord. Also following grafting cytokines might cross the semipermeable membrane of the graft, which would allow free diffusion of substances with a molecular weight lower that 70kDa. The study also found that the Fb/BDNF cells survived in the alginate capsules for a period of 1 month and continued to express the transgene, though down regulated and there was axon growth observed in the cavity region. The above experiment used a concentration of $3*10^6$ Fb/BDNF cells/ml of alginate and about 20-40 micro capsules were required to fill the lesion cavity. Previous studies by Liu *et al*, where Fb/BDNF were directly transplanted into the lesion, used a cell concentration of $5*10^4$ cells[106 and 107]. So

a.



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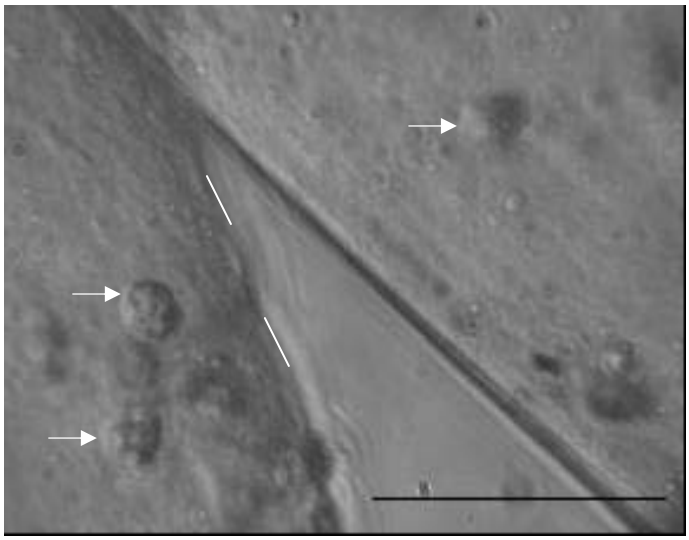


Figure 4.3.2a and b: Fb/BDNF in Modified Alginate String

Light micrographs showing Fb/BDNF in a piece of modified alginate string at 625X and 1000x respectively. The white dotted line represents the border of the string, the white arrows show the Fb/BDNF and the black line represents a scale of 100 μm

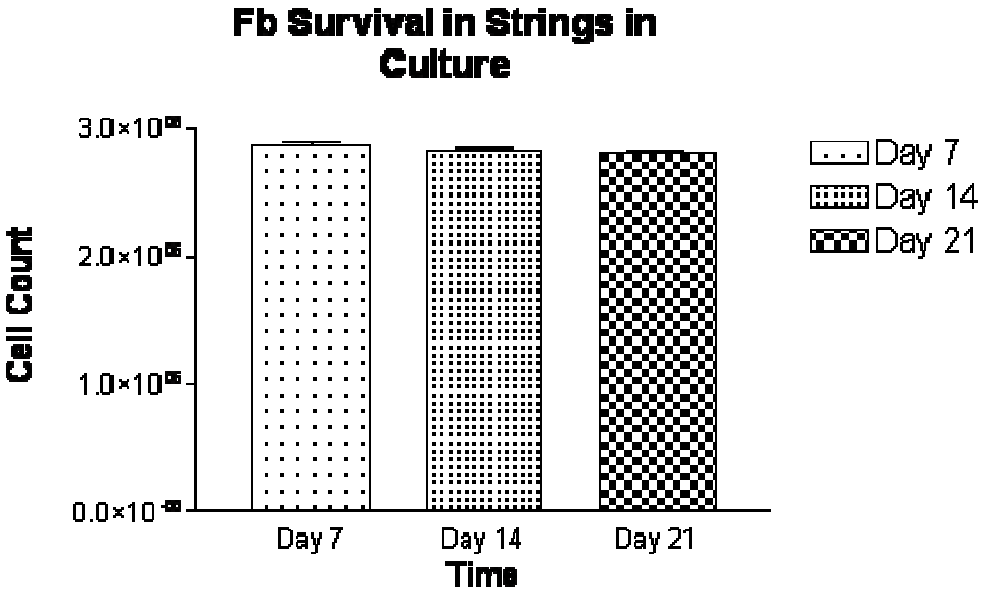
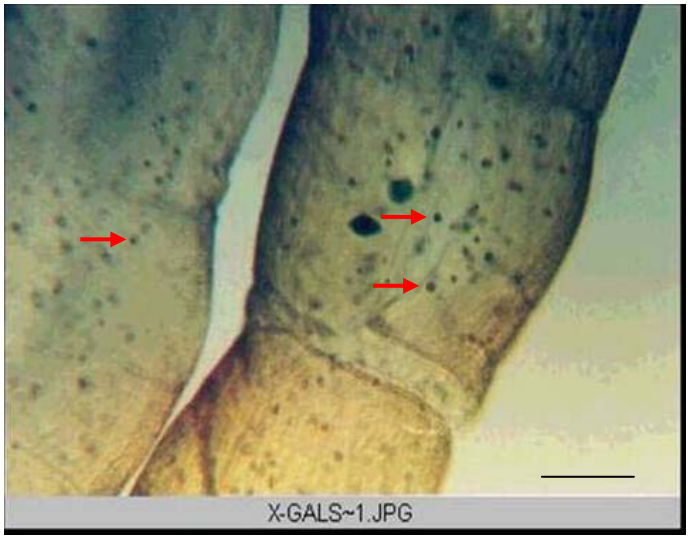


Figure 4.3.3: Fb/BDNF Cell Survival in Modified Alginate Strings, Suspended in Growth Medium, Over Time

This graph is a quantitative comparison of fibroblast survival, in equal volumes of alginate strings placed in growth media, at specific time points and n=3. The Y-error bars represent the standard error. The average cell count after 7 days was 2,858,333 (95.3%) , after 14 days was 2,825,000 (94.2%) and after 21 days was 2,802,000 (93.4%) of the initial cell concentration.

a.



b.

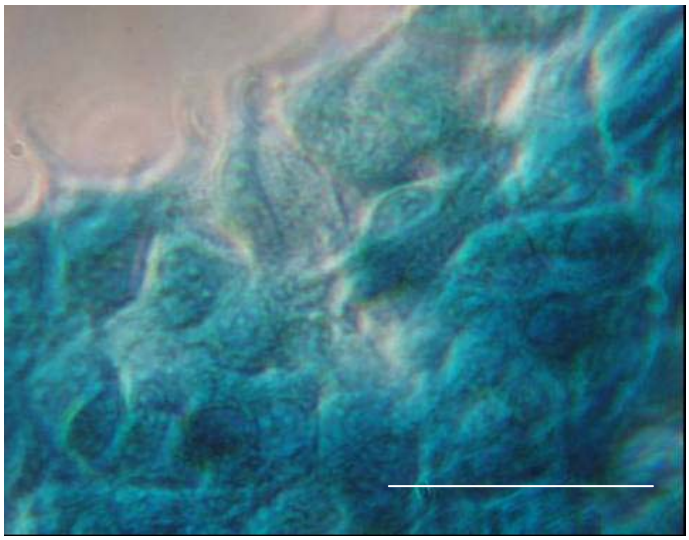


Figure 4.3.4a and b: X-Gal Staining of Fb/BDNF in Strings and when Released From Strings

The cells show a bluish green staining for the reporter gene. Figure a. is a light micrograph at 250X and b. Cells released by EDTA treatment viewed at 1000X. The arrows show the Fb/BDNF cells inside the strings. The size bars represent 100μm

depending on the size of the graft required the concentration of the cells has to be varied so as to produce approximately 10-20ng of BDNF/24 hours for a period of upto 1 month *in vivo* obtain desirable results.

4.3.3 *In vitro* Quantification of BDNF Secretion Using ELISA

The next step was to verify the concentration of BDNF secreted from the Fb/BDNF cells that were ensheathed in the modified alginate strings. This was done to see if the alginate string or the PLO coating or the peptide modified alginate played a role in influencing BDNF release. Experiments were set up with Fb/BDNF (at a concentration of $1*10^6$ cells in 1ml of alginate) in plain alginate strings, strings coated with PLO, strings coated with PLO and YIGSR modified alginate suspended in growth medium. Fb/BDNF ($1*10^6$ cells per well) cells in culture were used as controls. The growth medium was removed from the samples after 24 hours and ELISA was performed, as described in section 3.4.3, to estimate the concentration of BDNF released. The results in table 4.3.5 show the concentration of BDNF released from the different experimental samples. The results show that the Fb/BDNF ensheathed in alginate strings released about 8ng of BDNF per 24 hours while a similar number of free Fb/BDNF in culture released about 10ng per 24 hours. Growth medium, alginate strings, PLO and YIGSR peptide showed 0ng confirming

Table 4.3.1: BDNF Release from Fb/BDNF Cells Quantified by ELISA

SAMPLES	**BDNF CONCENTRATION (ng/24hours/million cells)**
Fb/BDNF in plain alginate strings	8.04
Fb/BDNF in plain alginate strings + PLO	7.33
Fb/BDNF in plain alginate strings + PLO + YIGSR modified alginate	7.45
Free Fb/BDNF in culture	9.97
Controls (Fb growth medium, plain alginate strings, strings + PLO, strings + PLO + YIGSR modified alginate)	0.0

that these factors did not influence the ELISA. Though there was a statistically significant difference ($p<0.001$) in BDNF secretion between the ensheathed and free Fb/BDNF there was no significant difference ($p>0.05$) in BDNF secretion from the cells in the strings with different coatings. The difference in BDNF concentration between the cells in the strings as opposed to cells in culture may be due to entrapment of BDNF in the alginate strings leading to a delay in its release. The release of the BDNF from the strings would be dependent on a number of factors such as the molecular weight of the alginate, the concentration of the calcium ions and the concentration of the peptide bound to the alginate which would all help to determine the pore size. Meanwhile the cells in culture would be unencumbered by any of these factors in their BDNF release. Previous experiments where Fb/ BDNF were directly transplanted in the lesion site, used cell concentrations ranging from $5*10^5$ cells to $3*10^6$ cells[112 and 113]. These experiments showed that the modified cells had maximum transgene expression in the first week of grafting and then the expression was subsequently down regulated, but when the cells were removed from the animal and placed in culture, they upregulated gene expression and continued to express the transgene for a period of at least 2 months when the animals were sacrificed. The experiments helped to prove that the cells were secreting sufficient

neurotrophic factors to aid in preventing neuronal atrophy and also influence neuronal regeneration. Varying the concentration of the cells added to the graft in the above mentioned concentrations seemed to produce similar results with regards to neuronal survival and regeneration. So depending on the size of the lesion which would determine the size of our graft, we could then vary the concentration of the Fb/BDNF cells/ml of alginate accordingly to ensure sufficient BDNF (minimum ~ 5ng/24 hours) release once grafted.

4.4 *In Vitro* evaluation of Frozen Modified Alginate Strings with Fb/BDNF

One of our aims was to evaluate storage of the graft so it would be readily available for transplant following injury. So we studied freezing as a method of storing the graft since it is a well established method of cell storage. Since there was no prior work on freezing grafts made of alginate we employed cell freezing protocol to study the effects of freezing the Fb/BDNF in modified alginate strings. Modified alginate strings made from equal volumes of alginate (1ml) with equal concentrations of Fb/BDNF ($1*10^6$ cells/ml) were placed in cryovials suspended in freezing media as described in section 3.4.2. *In vitro* evaluation was done to verify if the strings reconstituted on thawing. Other *in vitro* evaluation included studying the survival and transgene expression of Fb/BDNF from frozen modified alginate

strings.

4.4.1 Fb/BDNF Survival in Frozen Modified Alginate Strings

Modified alginate strings made from 1ml of alginate with $1*10^6$ Fb/BDNF were suspended in freezing medium and placed in cryovials which were in turn placed in a time delayed freezing container at -85°C for 24 hours before being transferred to liquid nitrogen. The strings were removed at specific time intervals and thawed at 37°C and dissolved in a 10% (w/v) EDTA solution. The cells were then resuspended in growth medium, centrifuged and stained with methylene blue to identify and count the viable cells. Figure 4.3.1 shows the results of cell survival over a year. The results show that nearly 85% of the cells ensheathed initially survived the long term freezing and thawing procedures. There was also no significant difference ($p>0.05$) in cell survival between the different time points. Since the protocol did no affect cell survival over prolonged storage under freezing the next step was to verify if the thawed Fb/BDNF still expressed the transgene.

4.4.2 X-Gal Staining of Fb/BDNF from Thawed Modified Alginate Strings

Fb/BDNF cells that were removed from frozen strings by treating with EDTA were stained for the reporter gene using the X-Gal staining kit. Cells that were

removed from the frozen strings were resuspended in growth medium in a culture plate for 24 hours. They were then stained using the X-gal staining kit to verify transgene expression. Figure 4.3.2 shows the bluish green staining of the cells for the reporter gene. The result confirms that the freezing medium helped in protecting the cells and the cells continued to express the transgene upon thawing when placed in culture.

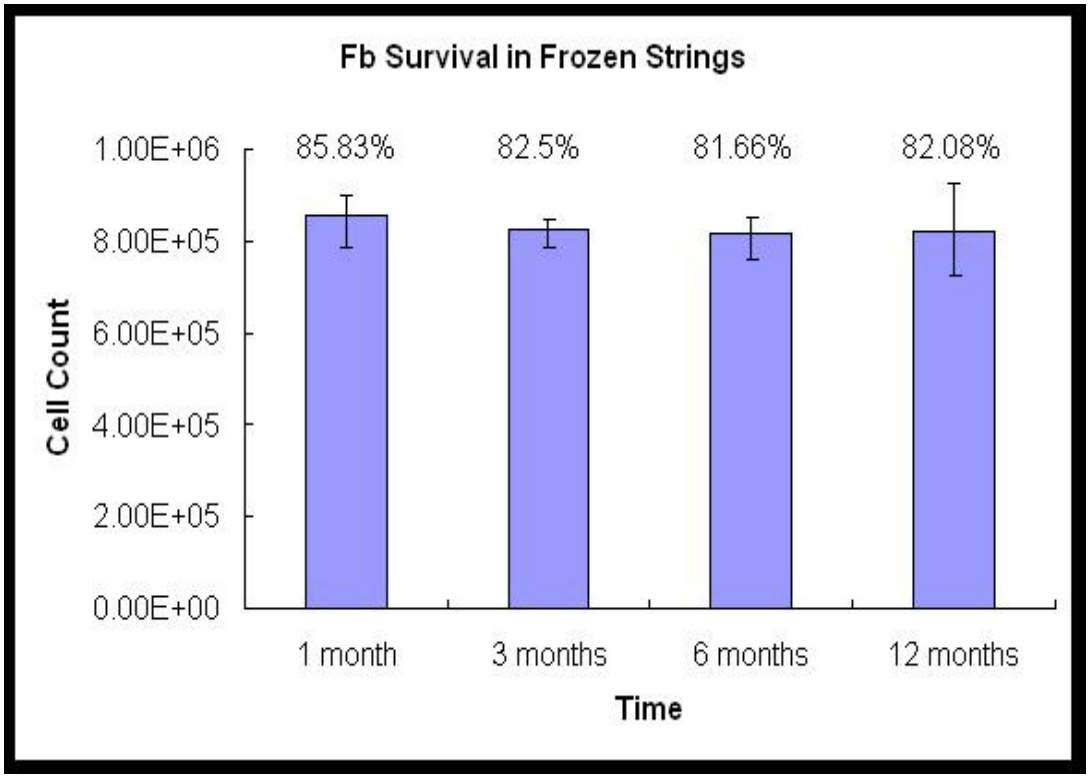


Figure 4.4.1: Fb/BDNF Cell Survival in Frozen Modified Alginate Strings
Results show that about 85% of the Fb/BDNF cells survive long term freezing. There is no significant difference ($p>0.05$) in cell survival between the various time points. The error bars represent the error over the mean.

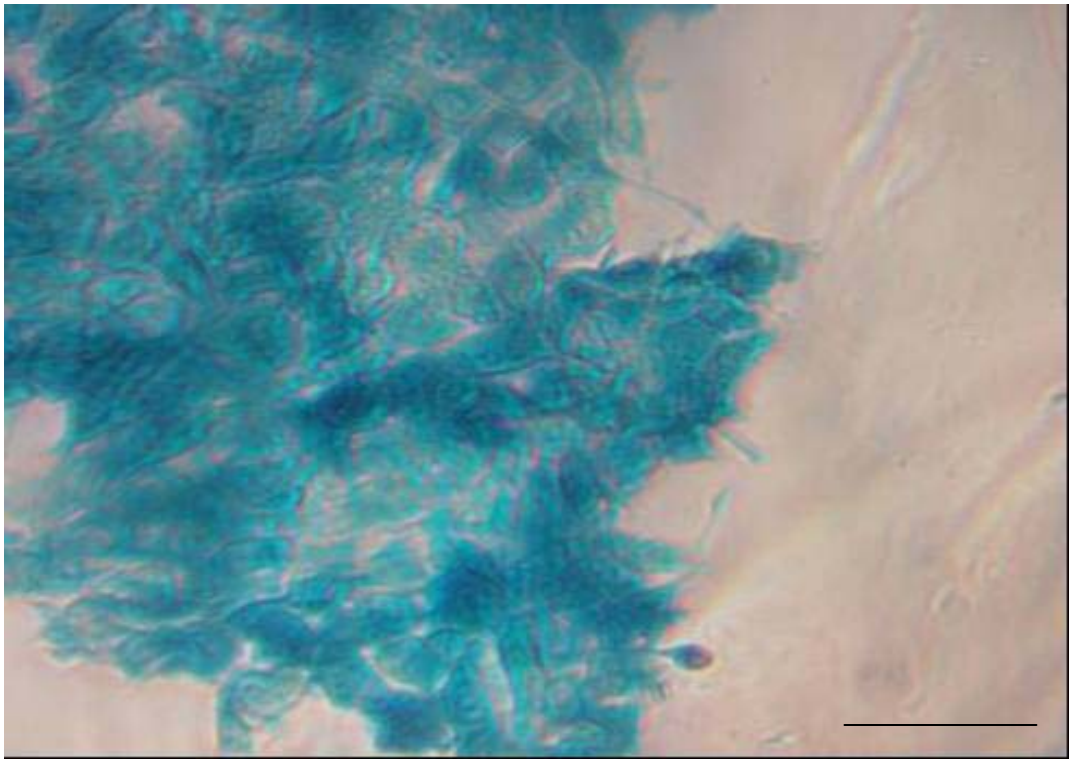


Figure 4.4.2: X-Gal Staining of Fb/BDNF Cells Released From Frozen Alginate Strings

The cells released from frozen alginate strings using EDTA that were thawed, showed bluish green staining for the reporter gene confirming that the cells were still actively expressing the BDNF transgene. The light micrograph was taken at a magnification of 250X and the size bar represents 100X.

4.5 Chick Embryo DRGs on Laminin Modified Alginate

To better mimic the effects of the graft *in vivo* we decided to use chick embryo dorsal root ganglia (DRGs). The rat and human neuroblastoma cell lines that were employed in the previous experiments were model cell lines. Thus these were undifferentiated cells and required specific differentiating factors for neurite extension. The DRGs are however differentiated cells and in the presence of medium with growth factors would start extending neurites *in vitro* that could be observed for a period of 48 hours. The DRGs require a laminin coated surface to adhere to before they could extend neurites[113]. The YIGSR pentapeptide sequence alone would not be sufficient for DRG adhesion since they require the entire laminin fragment. The initial idea was to coat alginate strings with laminin, but previous experiments performed by Dr. Dhoot with calcium alginate microcapsules proved that this method would be futile. When Ca-alginate microcapsules were coated with laminin the capsules started to fall apart even at concentrations as low as 1μg laminin per ml of alginate. This was due to the fact that laminin sequestered calcium from the calcium alginate complex leading to the disintegration of the hydrogel. The same was not observed in alginate hydrogels made with either barium or aluminum. This confirmed the results of other research that laminin formed large complexes

when it interacted with calcium ions[102]. So the next step was to covalently bind the laminin molecule to alginate utilizing carbodiimide chemistry as was the case with the pentapeptide YIGSR.

4.5.1 Cell Adhesion on Laminin Modified Alginate Discs and Strings

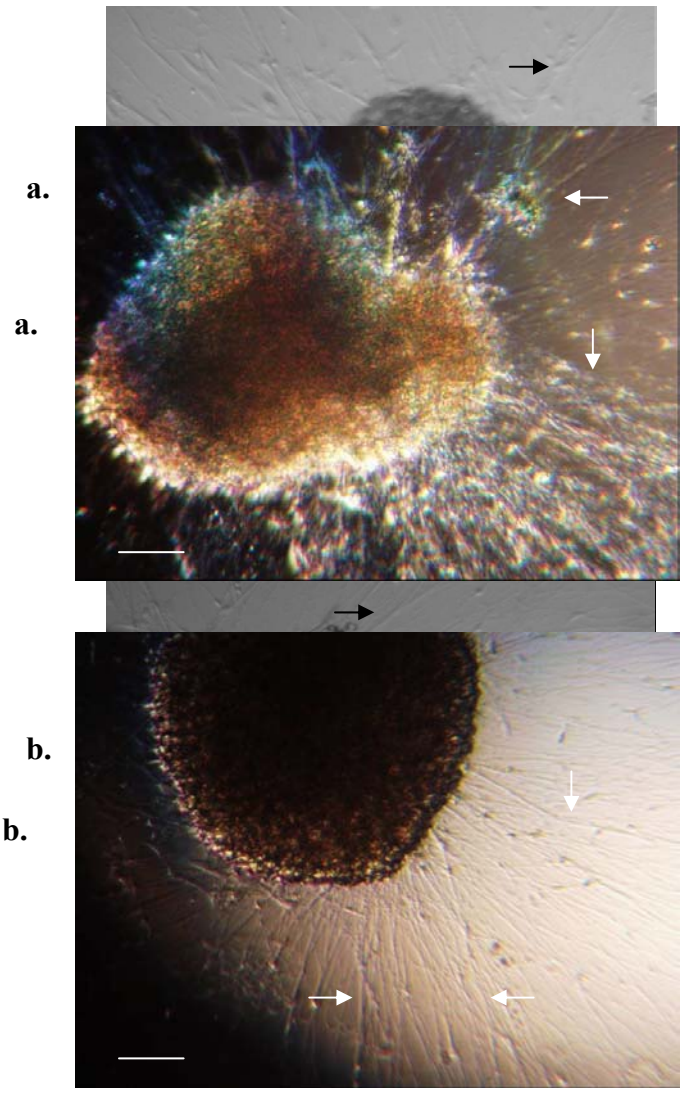
Alginate strings that were employed in prior experiments were found to be too small in width to allow for DRG adhesion since the DRGs were bigger than the NB2a and SHSY5Y cells that were employed. The alginate discs would provide a broader surface for the DRGs to adhere and extend neurites and help to verify the effectiveness of laminin modified alginate surface *in vitro*. Alginate discs were prepared using a $CaSO_4$ slurry[90] and then coated with PLO as with the strings. The discs were subsequently coated with alginate modified with laminin and washed repeatedly before they were used in cell adhesion experiments. DRGs removed from 10 - 12 day old chick embryos were placed on laminin modified alginate discs in 6 well culture plates and specific growth medium was added after 15 minutes. The discs in 3 wells were exposed to 1ml each of Fb growth medium removed from culture plate containing modified alginate strings, made from 1ml of alginate, with 10^6 Fb/BDNF (~10ng of BDNF/ 24 hours) cells ensheathed in them. The discs in the other 3 wells were exposed to 1ml of DRG growth medium which consists of

fibroblast growth medium supplemented with 10ng of human BDNF. A similar 6 well culture plate was also prepared with plain alginate discs in 3 wells that acted as controls, modified alginate discs with DRGs exposed to fibroblast growth medium without any BDNF supplement acted as controls in the other 3 wells. The culture plates were incubated at 37°C and pictures were taken under a microscope after 24 hours. DRG adhesion and neurite extension was then verified and compared.

The chick embryo DRGs adhere to the laminin modified alginate discs as seen in figures 4.5. The BDNF released into the medium, from the fibroblasts ensheathed in the modified alginate strings, induces the DRGs to produce neurites as seen in Figures 4.5.1a and 4.5.2a. DRGs on laminin modified alginate discs that were exposed to fibroblast growth media containing BDNF showed extensive neurite extension after 24 hours. DRGs on the control plate showed no neurite extension. Pictures were taken at 24 and 48 hours using an Olympus DP11 digital camera mounted on an inverted light microscope as well as a Nikon TE 2000 inverted light microscope as seen in Figure 4.5.3. The lengths of 30 random neurites on each DRG were measured, as shown in Figure 4.5.4, and a statistical analysis was performed. The average length of the neurites from DRGs exposed to fibroblast growth medium removed from culture plate with modified alginate strings with $1*10^6$ Fb/BDNF was

about 0.49mm after 24 hours and 0.88mm after 48 hours. The average length of neurites from DRGs exposed to fibroblast growth medium supplemented with 10ng of human BDNF was about 0.55mm after 24 hours and 0.92mm after 48 hours. DRG studies done by other scientists also show that the DRGs exposed to growth factors such as BDNF and NGF show neurite outgrowth at a maximum rate of 1 mm per 24 hours[114]. A statistical one way ANOVA test performed comparing the neurite extension from the two experimental sets shows that there is no statistically significant difference ($p>0.5$) in the average length of neurites between the DRGs exposed to growth medium removed from strings containing Fb/BDNF and DRGs exposed to BDNF supplemented growth medium at 48 hours. However at 24 hours the average length of the neurites from DRGs exposed to BDNF supplemented growth medium was significantly ($p<0.5$) longer than the average length of the neurites of the DRGs exposed to growth medium removed from culture plate containing modified alginate strings with Fb/BDNF. This helped to prove that the BDNF was being actively secreted by the ensheathed fibroblasts and most of the BDNF being released was being expressed outside the strings. DRGs placed on plain alginate discs did not adhere to the discs showing that alginate has no intrinsic cell adhesive properties.

The above experiments have helped to better mimic an *in vivo* environment and helped us to better understand how these grafts might perform in animal models. The next step would be to find a way to effectively bundle the strings with sterile sutures so they could be readily grafted into spinal injury models in animals.



Figures 4.5.1a & b: DRG on Laminin Modified Alginate Disc

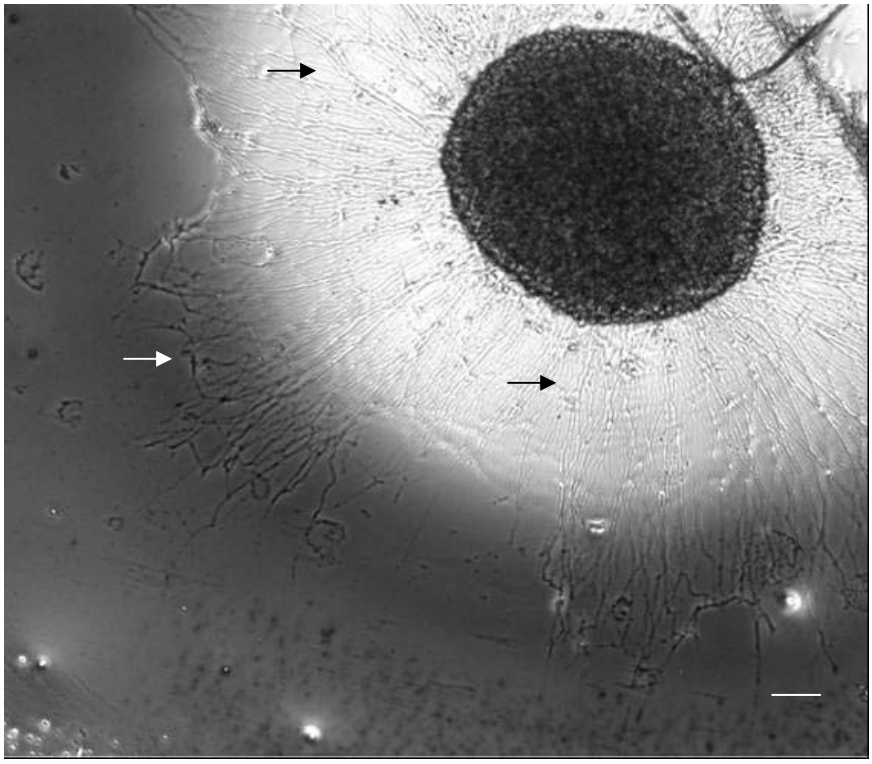


Figure 4.5.3: DRG on Laminin Modified Alginate Disc at 48 Hours

Shows a light micrograph of DRG on a laminin modified alginate disc taken at a magnification of 40X at 48 hours using a Nikon TE 2000 digital camera. Images taken under this microscope were used to measure neurite lengths. The arrows show the neurites and the white line at the bottom right represents a scale of 100μm

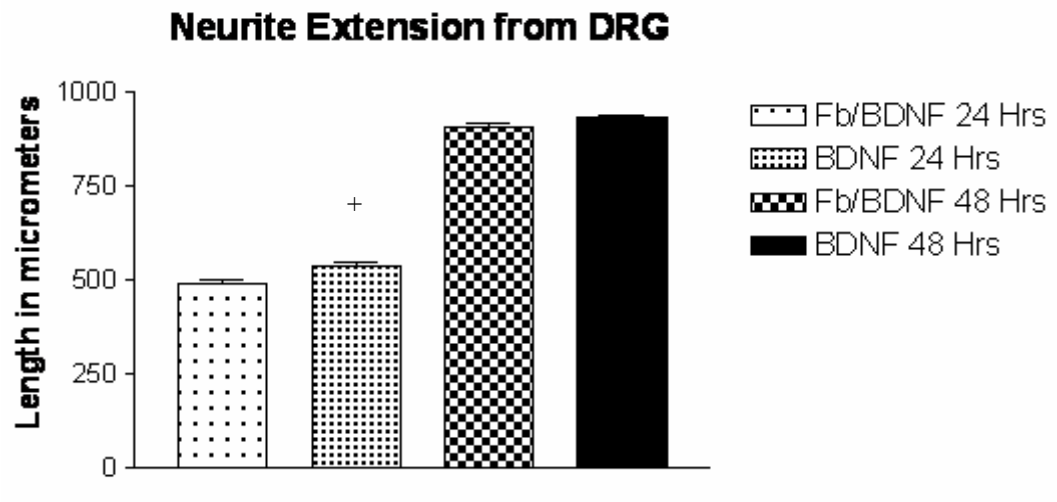


Figure 4.5.4: Average Length of Neurites Extending from DRGs Exposed to Different Media

This graph shows the average length of 30 randomly selected neurites from DRGs exposed to different media. Fb/BDNF stands for fibroblast growth media removed from culture plate containing laminin modified alginate strings with 10^6 Fb/BDNF cells ensheathed. BDNF stands for fibroblast growth media supplemented with 10ng of human BDNF. At 24 hours DRGs exposed to BDNF media showed significantly ($p<0.5$) longer neurites than those exposed to Fb/BDNF. At 48 hours there was no statistically significant difference in average neurite lengths between both groups. Y error bars represent standard error, n=30

4.5.2 Effectiveness of PLO Filter on Exposure to Anti-PMP Antibody

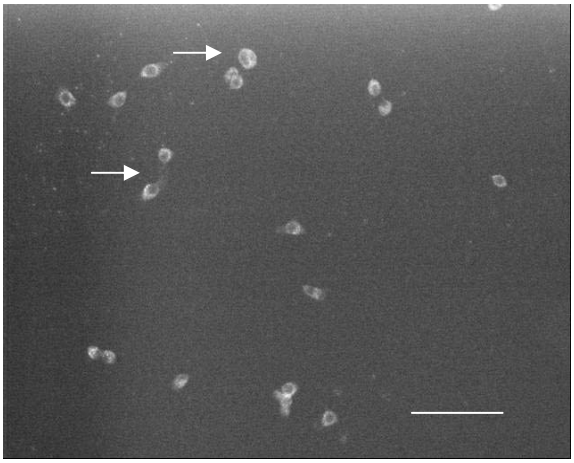
The function of the PLO coating is to prevent the high molecular weight immunoglobulins from entering the graft while allowing the Fb/BDNF to secrete the BDNF. While prior experiments with ELISA have proved that the

BDNF is being released from the graft, exposing the graft to anti-PMP 70 antibody would help to prove that the PLO filter is effective in guarding against immunoglobulins. The anti-PMP 70 antibody was chosen since it had a molecular weight of 70kDa which was the cut off for the semipermeable PLO filter and also it was a fluorescent antibody that would tag the cells making them clearly visible. Alginate strings, with 10^6 Fb/BDNF made from 1ml of alginate and coated with PLO and laminin modified alginate were placed in a 3 wells of a 12 well culture plate. A similar set of strings that had been frozen for 90 days were thawed in a 37°C water bath were placed in 3 wells. Plain alginate strings, with 10^6 Fb/BDNF made from 1ml of alginate without any coating were placed in 3 wells and a similar set of frozen strings were thawed and placed in the remaining 3 wells. The strings were then incubated for 2 hours at 37°C with 1µl of anti-PMP 70 antibody in 1 ml of buffer. The cells were then removed from the strings using 10% (w/v) sterile EDTA and fixed on cover slips using methanol free 16% (w/v) formaldehyde for 15

minutes. The cells were then exposed to Alexa Fluor 488 secondary antibody for 30 minutes under incubation. The cover slips were then observed under a Eclipse TE 2000U inverted fluorescent microscope (Nikon, Melville, NY) and pictures were captured using Cool Snap camera (Roper Scientific, Tucson, AZ).

Fb/BDNF in plain uncoated alginate strings, both freshly made and those that were frozen and then thawed took up the anti-peroxisomal membrane protein that they were exposed to as shown in Figures 4.6.1a and 4.6.2a. This is indicated by the fluorescence exhibited by the cells that have taken up the fluorescent secondary antibody. The Fb/BDNF in strings that were coated with PLO, both freshly made and those that were frozen and then thawed showed no fluorescence as seen in Figures 4.6.1b and 4.6.2b. The lack of fluorescence is due to the absence of the Alexa Fluor 488 secondary antibody uptake by the cells plated on the cover slips. This proved that the primary anti-PMP 70 antibody failed to penetrate the string showing that the PLO coating proved efficient. Similar effects were observed in strings that had been frozen for a period of 90 days and subsequently thawed in a 37°C water bath. This showed that the PLO coating on the strings was not compromised by the freezing and thawing procedures.

a.



b.

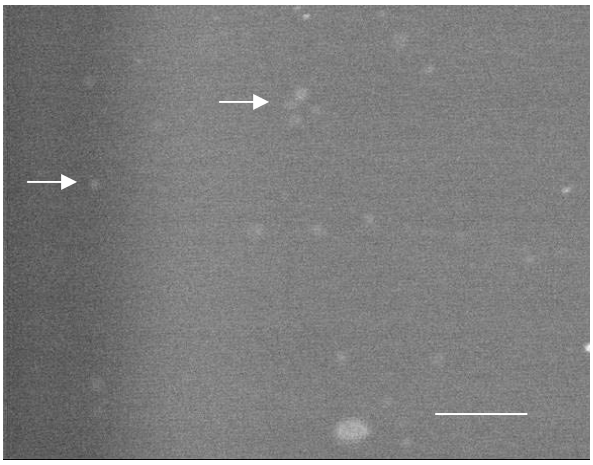
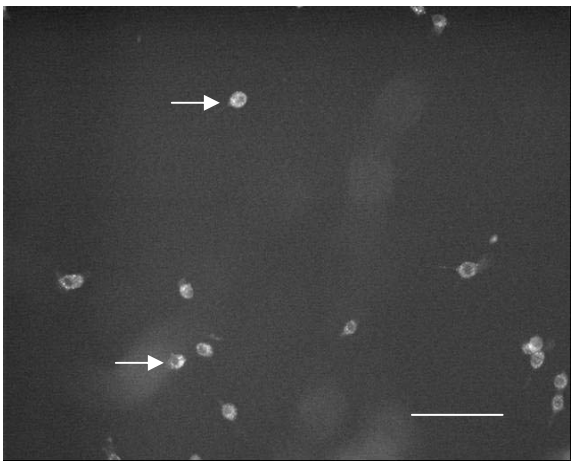


Figure 4.6.1a and b: Comparison of Antibody Uptake Between Cells Taken from Plain Alginate Strings and Strings Coated with PLO

Figure a. shows cells from plain alginate string that was exposed to anti-PMP antibody and fluorescent secondary antibody taken at a magnification of 250X. Figure b. shows cells from PLO coated alginate strings that were exposed to the antibodies taken at 250X. The white arrows show the cells. The white bar represents a scale of 100μm.

a.



b.

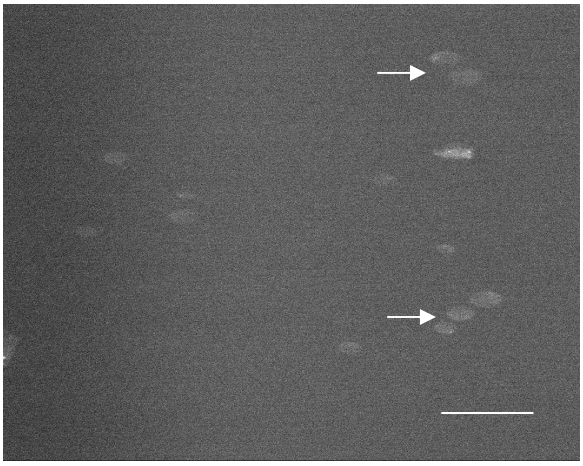


Figure 4.6.2a & b: Comparison of Antibody Uptake Between Cells Taken from Frozen Alginate Strings and Frozen Strings Coated with PLO

Figure a. shows cells from frozen and thawed plain alginate string that was exposed to anti-PMP antibody and fluorescent secondary antibody taken at a magnification of 250X. Figure b. shows cells from PLO coated frozen and thawed alginate strings that were exposed to the antibodies taken at 250X. The white arrows show the cells. The white bar represents a scale of 100μm.

5. CONCLUSIONS

This thesis evaluated the design and optimization of a novel graft in the form of alginate strings for use in treatment of spinal cord injury. The graft was designed to have a multifaceted action in aiding neuronal regeneration. Previous work and research in this field suggests the requirement for such treatment methods.

The graft for spinal injury treatment was designed in the form of alginate strings so that it could act as a bridge for neurons to grow across. Studies showed that the 22 gauge needle and a flow rate of 13.5ml per minute were optimum for producing alginate strings. The graft in the form of strings could act as a physical bridge at the site of injury, but alginate in itself has no inherent cell adhesive property. To enhance neuronal adhesion and regeneration alginate was modified with specific basement membrane peptide. YIGSR a pentapeptide of laminin was conjugated to alginate using carbodimide chemistry. Neuronal cells in the form of rat (NB2a) and human (SHSY5Y) neuroblastoma cells were used to verify cell adhesion. Early results showed that about 30% of the cells added to the graft adhered to it when alginate was modified with 1mg of YIGSR/ gm of alginate. Also NB2a cells showed better adhesion to the graft than SHSY5Y cells. Further studies to enhance cell adhesion showed that increasing the concentration of the peptide

conjugated to alginate helped to improve cell adhesion to about 45%. Maximum cell adhesion was attained with 2mg of YIGSR when 0.05 moles of the carboxyl groups of alginate were activated. These results led us to believe that more peptide could be conjugated to the alginate if higher concentrations of carboxyl groups were activated. Cell adhesion results confirmed that more peptide could be loaded if the concentration of carboxyl groups activated was increased. Maximum cell adhesion of about 70% was achieved with 3mg YIGSR per gm of alginate with about 0.4 moles of the carboxyl groups activated. The increase in the moles of carboxyl groups activated lead to an increase in the concentration of peptide that could be bound to alginate. The higher concentration of peptide helped to provide more adhesion sites thereby leading to an increase in the number of cells adhering to the modified alginate strings. The maximum percentage of cell adhesion could have been influenced by a number of factors like, maximum peptide that could be bound to alginate had been achieved, adhesion site crowding and high peptide concentration affecting cell adhesion.

The neuroblastoma cell lines that were employed in the adhesion studies were model cell lines and hence were undifferentiated. When medium consisting of specific differentiation factors was added to strings with the cells adhered to them,

the cells started to produce neurites. The most promising results were observed with the SHSY5Y cells at day 7, where many string samples showed cell body aggregation and neurite extension along the string. This further confirmed the fact that these modified alginate strings could act as a bridge along which the neurons could attach and produce neurites thereby helping the neurons to communicate across the gap at the injury site. The NB2a cells produced neurites that were short and did not extend along the string as the SHSY5Y cells. These cells were undifferentiated cells and required specific factors *in vitro* to produce neurites. Axon sprouting and growth on the graft may vary *in vivo* since the cells are differentiated and require no special factors.

Rat abdominal skin fibroblasts that were genetically engineered to express brain derived nerve growth factor were then ensheathed in these modified alginate strings. Experiments were performed to verify cell survival and transgene expression which showed that the fibroblasts in the strings survived in culture showing that the growth medium permeated into the strings. Unlike experiments were cells were encapsulated in alginate where the cells clumped together, there was no such phenomenon observed with the strings. The cell survival studies also showed no increase in cell count which may be due to the fact that the cells in the

center of the string could no communicate with the external environment thereby leading to their death due to lack of nutrients. The cells that were closer to the surface remained viable and reproduced maintaining a significant cell count. Staining for the reporter gene showed that the cells were still expressing the transgene while ensheathed in the alginate strings. These results were comparable to prior experiments done by Dr. Dhoot that showed that Fb/BDNF in alginate microcapsules continued to express the transgene *in vitro*. When the modified alginate strings with the Fb/BDNF are transplanted *in vivo* there might be a down regulation of transgene expression as experienced in studies with encapsulated Fb/BDNF. This would be due to exposure of the cells to cytokines that may pass through the semipermeable PLO coating and also due to the lack of exposure of the Fb/BDNF to the host tissue.

An enzyme linked immunosorbent assay was performed to quantify the BDNF released from the Fb/BDNF cells that were ensheathed in the strings. It was found that 10^6 cells released approximately 10ng of BDNF per 24 hours and the same concentration of cells in modified alginate strings released about 8ngs of BDNF. The BDNF release from strings that were coated with PLO and alginate modified with YIGSR showed similar release as the release from Fb/BDNF in plain alginate

strings. This showed that neither the YIGSR nor the PLO coating had any inhibiting effect on BDNF release from the graft. Fb/BDNF that were frozen in modified alginate strings also showed comparable BDNF release when thawed which confirmed the fact that freezing and thawing did not affect BDNF release. The results while showing that there was no drastic fall in BDNF secretion it also would aid in deciding the concentration of cell loading depending on the volume of alginate that would be required to make the graft.

The strings were frozen to make them readily available for grafting following injury. The modified alginate strings containing the Fb/BDNF were frozen using cell freezing protocols and cell survival, transgene expression and neuronal cell adhesion were verified. Experiments showed that approximately 85% of the Fb/BDNF cells survived freezing and continued to express the transgene. The freezing protocol used was the one used for cell storage and previous experiments on cryo preservation show cell survival of approximately 82-87%. This shows that this protocol could be used to store the graft. The strings also reconstitute on thawing thus making this process useful for storing the grafts for use when required. Neuronal cell adhesion was also found to be comparable to unfrozen strings showing that the YIGSR peptide was not affected by the freezing and thawing and continued to provide

adhesion sites for cell attachment.

The strings were then modified with the entire laminin fragment to support chick embryo dorsal root ganglion adhesion. These experiments were done to better mimic an in vivo situation since the neuroblastoma cells we employed earlier were undifferentiated cells. Laminin modified alginate discs and strings bundles were made for DRG adhesion experiments since they are bigger in diameter than a single strand of string. Experiments showed that DRGs adhered to modified alginate discs and also produced neurites when exposed to medium removed from strings with Fb/BDNF. The average length of neurites extending from DRGs exposed to medium supplemented with human BDNF was comparable to those that were exposed to medium removed from culture plated with modified alginate strings containing Fb/BDNF. Though there was statistically significant ($p<0.5$) difference in length between the two groups at 24 hours there was no significant ($p>0.5$) difference at 48 hours. This further helped to prove that the Fb/BDNF were surviving in the modified alginate strings and also actively expressing BDNF. The expressed BDNF also entered the surrounding medium thereby having a growth enhancing effect on the chick embryo DRGs. Previous studies have shown that 10^6 Fb/BDNF express about 10ng of BDNF per 24 hours and the above experiments have shown that the

average neurite length of the DRGs treated with human BDNF (10ng/ml) supplemented medium is comparable to those that are exposed to medium removed from culture plate with laminin modified alginate strings with Fb/BDNF expressing approximately the same concentration of BDNF. The difference in average neurite length between the two groups at 24 hours may be due to the fact that there may be an initial delay in the expression of the BDNF across the modified alginate strings into the surrounding medium. By 48 hours there is no significant difference in average neurite length thereby indicating that most of the BDNF expressed by the modified cells was released from the strings. The control experimental set up with unmodified alginate discs showed no DRG adhesion proving that alginate did not possess any intrinsic cell adhesive properties. The DRGs placed on laminin modified alginate discs, but exposed to fibroblast growth medium with no BDNF, showed adhesion but no neurite extension and this helped to prove that the medium had no effect on neurite extension *per se*.

In this thesis, it has been shown that alginate strings modified with specific peptide promote neuronal cell adhesion and growth. Cell adhesion could be enhanced by increasing the concentration of peptide bound to the alginate by in turn increasing the moles of carboxyl groups activated. The model cell lines that were

employed in early experiments showed that neuronal cells could adhere to the graft and produce neurites that would extend along the length of the graft. This also helped to show that this graft could act as a physical bridge *in vivo* for axons to grow across the lesion cavity and communicate. The alginate strings also contain genetically engineered cells that express neurotrophic factors that would further enhance neuronal regeneration. The graft has the ability to be stored by freezing making them readily available for use and have a PLO filter that would help to prevent host immune reaction from affecting the efficacy of the graft. This multifunctional graft could now be used for *in vivo* experiments in animals so that they could be optimized for use in humans.

6. RECOMMENDATIONS

Further studies are needed to further tune the work done in this thesis and the recommendations are presented below.

1. Optimize bundling of strings to keep the strands close together. This would help to enhance cell adhesion further. Keeping the strands close together would also help to keep the continuity of the graft. The strings should also be close together for the graft to act as a bridge at the injury site.
2. Study of the effect of BDNF released from the Fb/BDNF cells on chick embryo dorsal root ganglia. Add chick embryo DRGs to modified alginate strings and discs containing Fb/BDNF cells suspended in medium devoid of additional growth factors. Observe and quantitate neurite extension and length from the DRGs.
3. Study the effect of a growth factor gradient on neurite extension. Design a method to make modified alginate strings or discs with one half containing Fb/BDNF and the other half devoid of them. Place DRGs on these grafts and observe neurite extension. If the neurites extend towards the side with the Fb/BDNF then this design could be used to guide neurites at the injury site.

4. Study the effect of loading the enzyme chondroitinase ABC in modified alginate strings. The enzyme helps to degrade inhibitory proteoglycans that are present at the site of injury. A release profile would have to be performed to study the concentration of enzyme released from the strings. The effect of the enzyme on ensheathed Fb/BDNF cells on neuronal cell adhesion would also have to be verified. These would help to determine if the graft could have additional factors that could enhance neuronal regeneration at the injury site.
5. Study the concentration of peptide (YIGSR/Laminin) loading on the alginate. Experiments in this thesis have shown the maximum peptide loading in terms of cell adhesion, which is not necessarily the maximum concentration of peptide that could be bound to alginate. This might have to be verified by running the pure peptide through the high performance liquid chromatography (HPLC) and obtaining the range of absorbance and subsequently running the alginate-peptide combination.
6. Study the effects of varying alginate concentrations, calcium ion concentrations and peptide concentrations on BDNF release from the strings. Also check for BDNF release from strings modified with laminin and compare to strings modified with YIGSR. This would help to verify if BDNF release is better with

laminin modified strings or if the release would be inhibited by the large laminin molecule.

7. Finally study the effect of the graft in animal models to determine the effect of the graft *in vivo*. Place the modified alginate strings in rat spinal injury models and study the effects on neuronal regeneration by doing histopathalogical study on some animals and behavioral analysis on others. The histopathalogical studies would help to determine the type and length of neurites on the graft and the behavioral analysis would help to evaluate the limb functions in the paralyzed animals.

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Appendix A: Protocol for Bioconjugating YIGSR/Laminin to alginate

1. All glassware should be rinsed first with deionized water and then with alcohol and dried under a stream of argon.
2. Alginate (Manugel DMB, a high G-content alginate) is dissolved in MES buffer (0.1 M MES + 0.3 M NaCl, pH 6.5) to obtain a 1 (w/v) solution. The pH should be no higher than 6.5 since it affects the efficiency of the conjugation process using the carbodimide crosslinker.
3. Once the alginate is dissolved in the DI water 1-ethyl-3-(3-dimethylaminopropyl) carbodimide (EDC) is added to activate the carboxyl groups in alginate. Different molar concentrations of the carboxylic acid groups can be activated by varying the concentration of the EDC added.
4. N-hydroxysulfosuccinimide (sulfo-NHS) is then added at a molar ratio of 1:2 to EDC which help to stabilize the intermediate formed between EDC and the carboxylic acid groups of alginate.
5. The activation of the carboxyl acid groups is then allowed to continue for 15 minutes by continuously stirring the solution. The appropriate

concentration of either YIGSR or laminin is then added. The peptide conjugation process is then allowed to proceed for 24 hours by gently stirring the solution since vigorous stirring could lead to warming of the alginate solution and in turn hinder the peptide conjugation reaction.

6. Spectra/Por dialysis tubing is cut into several pieces and soaked in deionized water for 2 hours to remove any preservatives like sodium azide and glycerine. The water is replaced a few times to ensure complete removal of any preservatives from the tubing.

7. The alginate-peptide conjugate is then transferred to the cut and soaked pieces of dialysis of tubes and the tubes are sealed with closures.

8. The dialysis tubes with the modified alginate are then placed in a tank containing large volume of deionized water to remove any unreacted EDC, sulfo-NHS or peptide. This dialysis process is then allowed to continue for 4 days with the water being constantly replaced everyday to ensure complete removal of unreacted products.

9. The dialyzed alginate-peptide conjugate is then transferred to 50ml polypropylene centrifuge tubes and placed in a -85ºC freezer till the solution is completely frozen. The centrifuge tubes are then placed in a

-75ºC freeze dryer that has a vacuum of about 20 millitorrs, for about 4 days, which turns the alginate peptide conjugate into a dry fibrous form.

10. The alginate-peptide conjugate is weighed, labeled and then placed in a -20ºC freezer for future use.

Appendix B: Protocol for counting cells using a haemocytometer

1. Cells are removed from a culture plate by exposing them to 0.2% trypsin EDTA for 3 minutes. The enzyme helps to break the cell adhesion moieties that help the cells to adhere to the culture plate. The cells are not exposed to trypsin for more than 3 minutes to avoid the enzyme from digesting the cells.
2. After exposure to trypsin the cells are resuspended in growth medium containing fetal bovine serum to neutralize the action of trypsin and also to break up any cells that might still be adherent to the culture plate.
3. The cells suspended in growth medium are then placed in a 15ml centrifuge tube. The contents of the centrifuge tube are centrifuged at 1000 RPMs for 5 minutes. The cell pellet is then resuspended in growth medium to ensure complete removal of trypsin.
4. 20µl of growth medium with the cells suspended in it is placed in a 1.5ml microcentrifuge tube. 80µl of 0.4% Trypan blue stain is added to the cell suspension. The cells now form 1 in 5 parts of the solution. The

solution is then transferred to a hemocytometer using a pipette.

5. A cover slip is placed over the center of the hemocytometer and the cell suspension is added using capillary action by placing the tip of the pipette in the groove and just under the cover slip. The centre of the hemocytometer has 2 marked square chambers called Neubar's chamber.

6. The cells in the 4 large corner squares of the chamber, which has 16 small squares, are counted. The total count is then divided by 4 to get average cell count. Since each small square has a volume of 1μl the average cell count is then multiplied by 50,000 to get the total cell count in 1 ml of medium.

Appendix C: Calculations for preparation of YIGSR-alginate conjugate

Calculations were based on the molecular weight of the individual uronic acid units in alginate.

Reactant	Molecular Weight
Mannuronic/Guluronic Acid	194
EDC	191.7
Sulfo-NHS	217.1
YIGSR peptide	594.7

The number of uronic acid residues (carboxyl groups available for activation) in 1g of alginate were calculated to be $1/194 = 5.155 \times 10^{-3}$ moles.

Assuming that 1 mole of EDC would be required to activate 1 mole of carboxylic acid groups, the amount of EDC that would be required to activate carboxyl groups present in 1g of alginate would be 5.155×10^{-3} moles ~ 0.9882 g

Therefore the amount of EDC that would be required to activate 5% of the carboxylic acid groups present in 1g of alginate = 0.9882 g x 0.05 = 0.04947 ~ 50 mg (2.5775×10^{-4} moles of EDC)

Sulfo-NHS was added in the molar ration of 1:2 to EDC. Therefore the amount of sulfo-NHS added per g of alginate = (2.5775×10^{-4} moles x 217.1)/2 = 28mg

Number of moles of YIGSR peptide per mg = 0.001/594.7 = 1.6815×10^{-6} moles.

Appendix A

Protocol for Bioconjugating YIGSR/Laminin to alginate

1. All glassware should be rinsed first with deionized water and then with alcohol and dried under a stream of argon.
2. Alginate (Manugel DMB, a high G-content alginate) is dissolved in MES buffer (0.1 M MES + 0.3 M NaCl, pH 6.5) to obtain a 1 (w/v) solution. The pH should be no higher than 6.5 since it affects the efficiency of the conjugation process using the carbodimide crosslinker.
3. Once the alginate is dissolved in the DI water 1-ethyl-3-(3-dimethylaminopropyl) carbodimide (EDC) is added to activate the carboxyl groups in alginate. Different molar concentrations of the carboxylic acid groups can be activated by varying the concentration of the EDC added.
4. N-hydroxysulfosuccinimide (sulfo-NHS) is then added at a molar ratio of 1:2 to EDC which help to stabilize the intermediate formed between EDC and the carboxylic acid groups of alginate.
5. The activation of the carboxyl acid groups is then allowed to continue

for 15 minutes by continuously stirring the solution. The appropriate concentration of either YIGSR or laminin is then added. The peptide conjugation process is then allowed to proceed for 24 hours by gently stirring the solution since vigorous stirring could lead to warming of the alginate solution and in turn hinder the peptide conjugation reaction.

6. Spectra/Por dialysis tubing is cut into several pieces and soaked in deionized water for 2 hours to remove any preservatives like sodium azide and glycerine. The water is replaced a few times to ensure complete removal of any preservatives from the tubing.

7. The alginate-peptide conjugate is then transferred to the cut and soaked pieces of dialysis of tubes and the tubes are sealed with closures.

8. The dialysis tubes with the modified alginate are then placed in a tank containing large volume of deionized water to remove any unreacted EDC, sulfo-NHS or peptide. This dialysis process is then allowed to continue for 4 days with the water being constantly replaced everyday to ensure complete removal of unreacted products.

9. The dialyzed alginate-peptide conjugate is then transferred to 50ml polypropylene centrifuge tubes and placed in a -85ºC freezer till the

solution is completely frozen. The centrifuge tubes are then placed in a -75ºC freeze dryer that has a vacuum of about 20 millitorrs, for about 4 days, which turns the alginate peptide conjugate into a dry fibrous form.

10. The alginate-peptide conjugate is weighed, labeled and then placed in a -20ºC freezer for future use.

Appendix B

Protocol for counting cells using a hemocytometer

1. Cells are removed from a culture plate by exposing them to 0.2% trypsin EDTA for 3 minutes. The enzyme helps to break the cell adhesion moieties that help the cells to adhere to the culture plate. The cells are not exposed to trypsin for more than 3 minutes to avoid the enzyme from digesting the cells.
2. After exposure to trypsin the cells are resuspended in growth medium containing fetal bovine serum to neutralize the action of trypsin and also to break up any cells that might still be adherent to the culture plate.
3. The cells suspended in growth medium are then placed in a 15ml centrifuge tube. The contents of the centrifuge tube are centrifuged at 1000 RPMs for 5 minutes. The cell pellet is then resuspended in growth medium to ensure complete removal of trypsin.
4. 20µl of growth medium with the cells suspended in it is placed in a 1.5ml microcentrifuge tube. 80µl of 0.4% Trypan blue stain is added to

the cell suspension. The cells now form 1 in 5 parts of the solution. The solution is then transferred to a hemocytometer using a pipette.

5. A cover slip is placed over the center of the hemocytometer and the cell suspension is added using capillary action by placing the tip of the pipette in the groove and just under the cover slip. The centre of the hemocytometer has 2 marked square chambers called Neubar's chamber.

6. The cells in the 4 large corner squares of the chamber, which has 16 small squares, are counted. The total count is then divided by 4 to get average cell count. Since each small square has a volume of 1μl the average cell count is then multiplied by 50,000 to get the total cell count in 1 ml of medium.

VITA

Saravanan Kanakasabai was born in Chennai, Tamil Nadu in India on April 11 1974. He is an Indian citizen and attended Padma Seshadri Bala Bhavan Senior Secondary School (1977-89) and Sir M. Venkata Subba Rao Higher Secondary School (1989-91) for his primary and secondary school education. Following the completion of his secondary school education he attended Rajah Muthiah Medical College at Annamalai University (1991-1997) in pursuit of his Bachelor in Medicine and Bachelor in Surgery (M.B.B.S) Degree. He worked as an emergency room physician at Vijaya Health Center (1998-1999). He arrived at Drexel University in the Fall of 1999 in pursuit of his PhD in Biomedical Science and started as a research assistant at Dr. Wheatley's Microencapsulation and Drug Delivery lab in January 2000. Worked as a teaching assistant for 2 quarters in 2003 for the Body Synthetic, course. He received an award for poster presentation at the Northeast Bioengineering Conference in 2002 held at Drexel University. He was accepted for a podium presentation at the 2005 international BMES conference held at the Marriot in Philadelphia.